Document **AP1**
Appl. No. 09/722,441

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 0 754 756 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
**22.01.1997 Bulletin 1997/04**

(21) Application number: **95909969.8**

(22) Date of filing: **23.02.1995**

(51) Int. Cl.$^6$: **C12N 15/53**, C12P 13/08

(86) International application number:
**PCT/JP95/00268**

(87) International publication number:
**WO 95/23864 (08.09.1995 Gazette 1995/38)**

(84) Designated Contracting States:
**DE DK ES FR GB IT NL**

(30) Priority: **04.03.1994 JP 35019/94**

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# (54) PROCESS FOR PRODUCING L-LYSINE

(57) A coryneform bacterium having a high L-lysine productivity is provided by integrating a gene coding for a coryneform-origin aspartokinase released of feedback inhibition caused by L-lysine and L-threonine into a DNA of a chromosome of a coryneform bacterium carrying attenuated homoserine dehydrogenase or a coryneform bacterium deficient in a homoserine dehydrogenase gene.

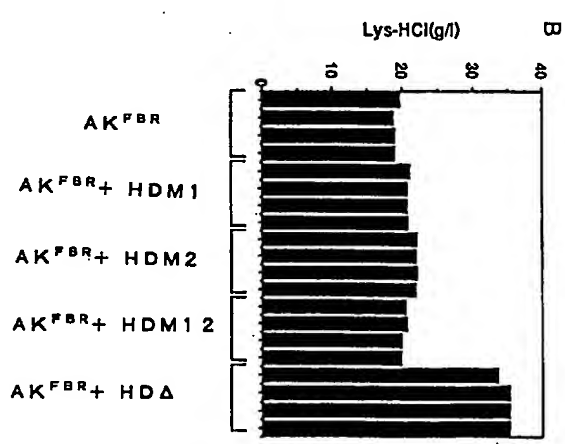


Fig. 7

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## Description

### Backgroud of the Invention

The present invention relates to microbial industry, and in particular relates to a method of producing L-lysine by fermentation, and coryneform bacteria preferable for use in this production method.

L-lysine has been hitherto produced by fermentation using L-lysine-producing bacteria belonging to the genus Brevibacterium, Corynebacterium, Bacillus or Escherichia, which is synthesized in a biosynthesis system of any of these microorganisms from oxaloacetate through aspartate, aspartate β-aldehyde and so on. Various enzymes such as phosphoenol pyruvate carboxylase, aspartokinase and dihydrodipicolinate synthase participate in such an L-lysine biosynthesis pathway, however, many of these enzymes undergo feedback inhibition by L-lysine as a final product or by aspartic acid as an intermediate product. Thus when L-lysine is produced by fermentation, in order to improve the productivity, many mutant strains which do not undergo such inhibition are used.

For example, it is known that aspartokinase (hereinafter referred to as "AK") undergoes concerted inhibition by L-lysine and L-threonine synthesized in a branched pathway from the L-lysine synthesis pathway in coryneform bacteria belonging to the genera such as Brevibacterium and Corynebacterium. A mutant strain harboring AK which does not undergo the inhibition is used for L-lysine production (J. Gen. Appl. Microbiol., 16, 373-391 (1970)).

A mutant strain, which lacks homoserine dehydrogenase (hereinafter referred to as "HD") considered to be an enzyme having the greatest influence on L-lysine productivity, is also used for production of L-lysine by fermentation. This is attributed to the fact that L-threonine is not synthesized due to deficiency in HD to catalyze a reaction for producing L-homoserine from aspartate β-semialdehyde as a first reaction in a synthesis pathway inherent to L-threonine branching from the L-lysine synthesis pathway through aspartate β-semialdehyde, resulting in progress of the L-lysine synthesis reaction without inhibition of the AK activity. As such an HD deficient strain, an HD completely deficient strain of Corynebacterium glutamicum is known (Nakayama, K. et al.; J. Gen. Appl. Microbiol. 7(3), 145-154 (1961)).

In addition to the HD completely deficient strain as described above, a mutant strain harboring so-called leaky type HD is considered to be effective for L-lysine production, as well. The HD completely deficient strain cannot synthesize L-threonine and L-methionine, and thus it cannot grow unless these amino acids are present in a medium. On the contrary, if an HD leaky type strain can be obtained that harbors a leaky type HD which does not substantially exhibit activity so much to suppress L-lysine production but has HD activity a little, it becomes possible to make growth without addition of L-threonine and L-methionine to a medium, and it becomes convenient to prepare the medium.

Additionally, the leaky type HD has small affinity to aspartate β-semialdehyde as its substrate. Therefore, the HD leaky type strain synthesizes a considerable amount of aspartate β-semialdehyde for synthesizing L-threonine, L-methionine and L-isoleucine required for the growth. Aspartate β-semialdehyde synthesized in a considerable amount is consequently converted into L-lysine.

On the other hand, the HD completely deficient strain is considered to be still useful in that it completely suppresses production of L-threonine in amount, however, the deficiency in HD as a result of mutation has a possibility to restore the activity due to reverse mutation. Thus an HD deficient strain, in which such a possibility is extremely low with a destroyed HD gene, is considered to be more useful. A nucleotide sequence of an HD gene has been reported by Peoples et al. for Corynebacterium glutamicum (Peoples, O. P. et al., Molecular Microbiology, 2(1), 63-72 (1988)).

Since the HD leaky type strain and the HD deficient strain do not produce L-threonine, AK does not undergo feedback inhibition. Accordingly, it is expected that if the AK gene is amplified in cells of the HD leaky type strain and the HD deficient strain, the L-lysine synthesis reaction proceeds, and the L-lysine productivity is improved. It is further expected that L-lysine productivity is more improved by introducing, into coryneform bacteria, mutation of AK to avoid feedback inhibition by L-lysine and L-threonine in combination with leakage or deficiency of HD.

### Disclosure of the Invention

The present invention has been made taking the aforementioned viewpoints into consideration, a task of which is to obtain an HD leaky type strain and an HD gene-destroyed strain, and provide an HD leaky type strain and an HD deficient strain with an amplified AK gene, and an HD leaky type strain and an HD gene-destroyed strain harboring AK which does not undergo feedback inhibition by L-lysine and L-threonine, in order to improve the L-lysine productivity of coryneform bacteria.

In order to solve the aforementioned task, the present inventors have obtained an HD leaky type mutant strain of Brevibacterium lactofermentum, isolated a wild type HD gene and a leaky type HD gene to clarify their structures, introduced the leaky type HD gene and a partially deleted HD gene into a wild strain of Bravibacterium lactofermentum, and thus created an L-lysine-producing strain having improved L-lysine productivity. The present inventors have succeeded in further improvement in L-lysine productivity by amplifying an AK gene in cells of the L-lysine-producing strain thus obtained, or by introducing a gene coding for AK which does not undergo feedback inhibition by L-lysine and L-threonine, and arrived at the present invention.

Namely, the present invention provides a DNA fragment which codes for homoserine dehydrogenase originating from a coryneform bacterium in which at least one of a 23rd leucine residue and a 104th valine residue as counted from its N-terminus is changed to another amino acid residue; a coryneform bacterium which harbors a gene coding for mutant homoserine dehydrogenase originating from a coryneform bacterium in which at least one of a 23rd leucine residue and a 104th valine residue as counted from its N-terminus is changed to another amino acid residue; and a coryneform bacterium which is transformed by integrating the aforementioned gene coding for mutant type homoserine dehydrogenase into chromosomal DNA by way of homologous recombination with a homoserine dehydrogenase gene on a chromosome of the coryneform bacterium.

In another aspect, the present invention provides a coryneform bacterium wherein its homoserine dehydrogenase gene is destroyed by integrating a DNA fragment coding for a part of homoserine dehydrogenase originating from a coryneform bacterium into chromosomal DNA by way of homologous recombination with a homoserine dehydrogenase gene on a chromosome of the coryneform bacterium. In still another aspect, the present invention provides a coryneform bacterium which harbors in its cells recombinant DNA constructed by ligating an aspartokinase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of coryneform bacteria, and expresses no wild type homoserine dehydrogenase; and a coryneform bacterium which is transformed by integrating, into chromosomal DNA of the coryneform bacterium, a gene coding for aspartokinase originating from a coryneform bacterium with desensitized feedback inhibition by L-lysine and L-threonine, and expresses no wild type homoserine dehydrogenase. In still another aspect, the present invention provides a method of producing L-lysine comprising the steps of cultivating the coryneform bacterium described above in an appropriate medium, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture.

In this specification, occasionally, a strain which produces wild type HD or wild type AK is referred to as "wild strain", HD having the leaky type mutation which scarcely exhibits substantial HD activity but has HD activity a little is merely referred to as "mutant type HD", AK having mutation not to undergo feedback inhibition by L-lysine and L-threonine is referred to as "mutant type AK", and a partially deleted HD gene is referred to as "deletion type HD gene". Further, the integration of recombinant DNA comprising a foreign HD gene or a foreign AK gene and a vector into chromosomal DNA by way of homologous recombination with an HD gene or an AK gene on host chromosomal DNA is referred to as "gene integration", and the achievement of a state in which the HD gene or the AK gene on the chromosome is replaced by the foreign HD gene or the foreign AK gene by allowing one copy of an HD gene or an AK gene to fall off together with the vector from a state in which the recombinant DNA is integrated into the chromosomal DNA is referred to as "gene replacement". Furthermore, a mutant strain harboring a mutant type HD gene or a strain subjected to gene replacement with a mutant type HD gene is merely referred to as "HD mutant strain", and a strain subjected to gene replacement with a partially deleted HD gene is referred to as "HD deficient strain", as well.

The coryneform bacteria referred to in the present invention is a group of microorganisms defined on page 599 in "Bergey's Manual of Determinative Bacteriology", eighth edition (1974), which reside in aerobic Gram-positive non-acid-fast rods having no spore-forming ability, including bacteria belonging to the genus Corynebacterium, bacteria belonging to the genus Brevibacterium having been hitherto classified into the genus Brevibacterium but united as bacteria belonging to the genus Corynebacterium at present, and bacteria belonging to the genus Brevibacterium closely relative to bacteria belonging to the genus Corynebacterium.

The HD mutant strain obtained according to the present invention is excellent in L-lysine productivity, and it can grow even when L-methionine and L-threonine, or L-homoserine is absent in a medium. The HD deficient strain of the present invention is excellent in L-lysine productivity because of no expression of the HD gene, and it can stably maintain this property.

Further, the HD mutant strain and the HD deficient strain with amplified AK gene, as well as the HD mutant strain and the HD deficient strain harboring the mutant type AK gene are more excellent in L-lysine productivity.

## Detailed Description of the Invention

The present invention will be explained in detail below.

### (1) Preparation of leaky type HD mutant strain and mutant type HD gene

A mutant strain which produces HD having leaky type mutation is obtained by performing a mutation treatment of a coryneform bacterium which produces wild type HD. For the mutation treatment of the coryneform bacterium, a treatment is conducted by using ultraviolet light irradiation or a mutating agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosog uanidine (NTG).

Bacterial cells after the mutation treatment were subjected to single colony isolation to select those producing the leaky type HD from each of colonies. Leaky type HD mutant strains can grow on a minimum medium, cannot grow on a minimum medium added with excessiv L-methionine and L-threonine, but can grow on a minimum medium added with L-homoserine, or L-methionine and L-threonine. Thus they can be selected using the foregoing as a criterion

(Shiio, I. & Sano, K., J. G. A. M., 15, 267-287 (1969)). In order to confirm the fact that mutant strains thus obtained produce the leaky type HD, it is preferable to extract a crude enzyme solution from bacterial cells and compare the HD specific activity with that of wild type HD.

The enzyme activity of HD can be measured in accordance with, for example, a method of Kalinowski et al. (Kalinowski, J. et al., Mol. Gen. Genet., 224, 317-324 (1990)) using a crude enzyme solution prepared from bacterial cells in accordance with a method of Follettie et al. (Follettie, M. T. et al., Molecular Microbiology, 2, 53-62 (1988)).

In order to isolate the mutant type HD gene from the obtained leaky type HD mutant strains, chromosomal DNA is prepared from the leaky type HD mutant strains in accordance with, for example, a method of Saito and Miura (H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963)), and the HD gene is amplified by means of a polymerase chain reaction method (PCR; see White, T. J. et al., Trends Genet., 5, 185 (1989)). For DNA primers to be used for the amplification reaction, those complementary to both 3' terminals of a DNA double strand containing an entire or partial region of the HD gene are used. When only a partial region of the HD gene is amplified, it is necessary to screen a DNA fragment containing an entire region from a chromosomal DNA library using such DNA fragments as primers. When an entire region of the HD gene is amplified, a PCR reaction solution containing a DNA fragment including the amplified HD gene is subjected to agarose gel electrophoresis, followed by extraction of the objective DNA fragment. Thus the DNA fragment containing the HD gene can be recovered.

DNA primers may be appropriately prepared, for example, on the basis of a sequence known for Corynebacterium glutamicum (Peoples, O. P. et al, Molecular Microbiology, 2(1), 63-72 (1988)). Specifically, primers which can amplify a region comprising 1150 base pairs coding for the HD gene are preferable, and for example, two species of primers defined with SEQ ID NOS: 1 and 2 are suitable. The primer DNA can be synthesized in accordance with an ordinary method such as a phosphoamidite method (see Tetrahedron Letters, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using Taq DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designated by the supplier.

It is preferable for the mutant type HD gene amplified by the PCR method to be connected to vector DNA autonomously replicable in cells of Escherichia coli (hereinafter referred to as "E. coli", as well) and/or coryneform bacteria to prepare recombinant DNA which is introduced into E. coli cells, in order to facilitate following operations. The vector autonomously replicable in cells of E. coli is preferably a plasmid vector, preferably as those autonomously replicable in cells of a host, including, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, and RSF1010.

It is preferable that such a vector is inserted with a DNA fragment having an ability to make the plasmid autonomously replicable in coryneform bacteria which can be prepared, for example, from plasmids pAM330 (see Japanese Patent Laid-open No. 58-67699), pHM1519 (see Japanese Patent Laid-open No. 58-77895), pCG1 (see Japanese Patent Laid-open No. 57-134500), pCG2 (see Japanese Patent Laid-open No. 58-35197), pCG4 (see Japanese Patent Laid-open No. 57-183799), and pCG11 (see Japanese Patent Laid-open No. 57-183799). Thus the vector can be used as so-called shuttle vector which is autonomously replicable in both of E. coli and coryneform bacteria.

Such a shuttle vector is exemplified by the followings. Microorganisms harboring each of the vectors and deposition numbers of international deposition institutes are indicated in parentheses.

| | |
|---|---|
| pAJ655: | Escherichia coli AJ11882 (FERM BP-136) |
| | Corynebacterium glutamicum SR8201 (ATCC 39135) |
| pAJ1844: | Escherichia coli AJ11883 (FERM BP-137) |
| | Corynebacterium glutamicum SR8202 (ATCC 39136) |
| pAJ611: | Escherichia coli AJ11884 (FERM BP-138) |
| pAJ3148: | Corynebacterium glutamicum SR8203 (ATCC 39137) |
| pAJ440: | Bacillus subtilis AJ11901 (FERM BP-140) |

These vectors are obtained from deposited microorganisms as follows. Cells collected at the logarithmic growth phase are lysed with lysozyme and SDS to give a lysate from which a supernatant solution is obtained by centrifugation at 30,000 x g. Polyethylene glycol is added to the supernatant solution to perform fractional purification by means of cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

In order to introduce a plasmid into E. coli for transformation, it is possible to use, for example, a method of D. M. Morrison (Methods in Enzymology, 68, 326 (1979)), or a method for treating recipient cells with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)).

When the mutant type HD gene is isolated from the leaky type HD mutant strain, it is also obtained by preparing a chromosomal DNA library from the leaky type HD mutant strain using a plasmid vector or the like, selecting a strain harboring the mutant type HD gene from the library, and recovering recombinant DNA with the inserted mutant type HD gene from the selected strain. An example of a method for preparing the chromosomal library and selecting the strain harboring the mutant type HD gene from the library will be described below.

At first, a leaky type HD mutant strain is cultivated to obtain a culture. Any medium in which coryneform bacteria can grow is available for use. When L-threonine and L-methionine are contained in the medium in small amounts, it is preferable to add L-threonine and L-methionine, or L-homoserine beforehand. Next, bacterial cells are obtained by centrifuging the culture. Chromosomal DNA is obtained from the bacterial cells in accordance with, for example, a method of Saito and Miura (Biochem. Biophys. Acta, 72, 619 (1963)) or a method of K. S. Kirby (Biochem. J., 64, 405 (1956)).

In order to isolate the mutant type HD gene from the chromosomal DNA thus obtained, a chromosomal DNA library is prepared. At first, the chromosomal DNA is partially decomposed with an appropriate restriction enzyme to obtain a mixture of various fragments. A wide variety of restriction enzymes can be used by controlling the degree of cutting by controlling a period of time of the cutting reaction or the like. For example, Sau3AI is allowed to act on the chromosomal DNA to digest it at a temperature of not less than 30°C, preferably at 37°C at an enzyme concentration of 1-10 units/ml for various periods of time (1 minute to 2 hours).

Subsequently, the cut chromosomal DNA fragments are ligated with a vector autonomously replicable in E. coli cells to prepare recombinant DNA. Specifically, a restriction enzyme, which generates the same terminal nucleotide sequence as that by the restriction enzyme Sau3AI used for cutting the chromosomal DNA, for example, BamHI is allowed to act on the vector DNA to completely digest it under conditions of a temperature of not less than 30°C and an enzyme concentration of 1-100 units/ml for not less than 1 hour, preferably for 1-3 hours, to achieve cutting and cleavage. Next, the chromosomal DNA fragment mixture obtained as described above is mixed with the cleaved and cut vector DNA, on which DNA ligase, preferably T4 DNA ligase is allowed to act under conditions of a temperature of 4-16°C and an enzyme concentration of 1-100 units/ml for not less than 1 hour, preferably for 6-24 hours, to obtain recombinant DNA.

Using the obtained recombinant DNA, for example, E. coli K-12 strain is transformed to prepare a chromosomal DNA library. The transformation may be performed by, for example, a method of D. M. Morrison (Methods in Enzymology, 68, 326 (1979)), or a method for treating recipient cells with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)).

In order to select a transformant strain harboring the mutant type HD gene from the obtained chromosomal DNA library, for example, an oligonucleotide probe may be synthesized on the basis of a sequence known for Corynebacterium glutamicum (Peoples, O. P. et al., Molecular Microbiology, 2(1), 63-72 (1988)), to perform colony hybridization using it. It is known that two kinds of E. coli HD genes (HD-1, HD-2) are present (Zakin, M. M. et al., J. B. C., 258, 3028-3031 (1983)), however, any of them has no region corresponding to about 100 amino acid residues on the C-terminal side of Corynebacterium glutamicum HD. Thus when a sequence to be used for the probe is selected from this region, it does not hybridize to the HD gene on E. coli chromosome, which is preferable. Recombinant DNA containing the mutant type HD gene can be isolated from transformed strains thus selected in accordance with, for example, a method of P. Guerry et al. (J. Bacteriol., 116, 1064 (1973)) or a method of D. B. Clewell (J. Bacteriol., 110, 667 (1972)).

Alternatively, a strain which produces the leaky type HD may be created by using a wild type HD gene cloned from a coryneform bacterium in the same manner as described above. At first, DNA containing a wild type HD gene or an HD gene having another mutation is subjected to an in vitro mutation treatment, and DNA after the mutation treatment is ligated with vector DNA adapted to a host to obtain recombinant DNA. The recombinant DNA is introduced into a host microorganism to obtain transformants, and one which expresses the leaky type HD is selected from the transformants. Alternatively, it is also available that DNA containing a wild type HD gene or an HD gene having another mutation is ligated with vector DNA adapted to a host to obtain recombinant DNA, thereafter the recombinant DNA is subjected to an in vitro mutation treatment, the recombinant DNA after the mutation treatment is introduced into a host microorganism to obtain transformants, and one which expresses the leaky type HD is selected from the transformants.

The agent for performing the in vitro mutation treatment of DNA is exemplified by hydroxylamine. Hydroxylamine is a treatment agent for chemical mutation which causes mutation from cytosine to thymine by changing cytosine to $N^4$-hydroxycytosine.

The mutant type HD gene for use in the present invention is not especially limited provided that it codes for the leaky type HD, for which there are exemplified genes coding for HD having any of mutations in an amino acid sequence of wild type HD, including:

(1) mutation to change a 23rd leucine residue from the N-terminal to an amino acid residue other than the leucine residue;
(2) mutation to change a 104th valine residue from the N-terminal to an amino acid residue other than the valine residue; and
(3) mutation to change the 23rd leucine residue from the N-terminal to an amino acid residue other than the leucine residue, and the 104th valine residue from the N-terminal to an amino acid residue other than the valine residue.

The amino acid sequence of wild type HD is herein specifically exemplified by an amino acid sequence of HD originating from a wild type strain of Brevibacterium lactofermentum shown in SEQ ID NO: 3 and SEQ ID NO: 4 in Sequence Listing.

With respect to the mutations described in the foregoing (1) to (3), mutation to change to a phenylalanine residue is exemplified for the 23rd leucine residue, and mutation to change to an isoleucine residue is exemplified for the 104th valine residue.

Any codon corresponding to the replaced amino acid residue is available especially regardless of its type provided that it codes for the amino acid residue. The amino acid sequence of harbored wild type HD may slightly differ depending on difference in bacterial species and bacterial strains. HD having such replacement, deletion or insertion of amino acid residues at positions irrelevant to the activity of the enzyme can be also used for the present invention.

For example, as will be described in Examples below, as a result of comparison of an amino acid sequence of HD originating from Brevibacterium lactofermentum 2256 strain (ATCC 13869) with an amino acid sequence reported for HD of Corynebacterium glutamicum (Peoples, O. P. et al., Molecular Microbiology, 2(1), 63-72 (1988)), it has been clarified that a 148th amino acid residue from the N-terminal is a glycine residue in HD of Corynebacterium glutamicum, while it is an alanine residue in HD of Brevibacterium lactofermentum. It is expected that the leaky type HD is obtained by introducing any of the aforementioned mutations (1) to (3) even in the case of HD of Corynebacterium glutamicum as described above.

(2) Preparation of wild type AK gene and mutant type AK gene

The wild type AK gene for use in the present invention can be prepared from wild strains of coryneform bacteria. A gene, which codes for AK in which cumulative feedback inhibition by L-lysine and L-threonine is substantially desensitized, can be prepared from a mutant strain in which cumulative feedback inhibition to the AK activity by L-lysine and L-threonine is substantially desensitized. Such a mutant strain can be obtained from a group of cells having been subjected to a mutation treatment applied to, for example, a wild strain of coryneform bacteria by using an ordinary mutation treatment method such as ultraviolet light irradiation or a treatment with a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanid ine (NTG). For measuring the AK activity, it is possible to use a method described by Miyajima, R. et al. in The Journal of Biochemistry (1968), 63(2), 139-148.

With respect to donor bacteria for the AK gene, a wild strain ATCC 13869 of Brevibacterium lactofermentum, and an L-lysine-producing bacterium AJ3463 (FERM P-1987) derived from the ATCC 13869 strain by a mutation treatment are most preferable donor bacteria.

In order to isolate the AK gene from the coryneform bacteria, chromosomal DNA is prepared in accordance with, for example, a method of Saito and Miura (H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963)), and the AK gene is amplified by means of a polymerase chain reaction method (PCR; see White, T. J. et al., Trends Genet., 5, 185 (1989)).

For DNA primers to be used for the amplification, those complementary to both 3' terminals of a DNA double strand containing an entire or partial region of the AK gene are used. When only a partial region of the AK gene is amplified, it is necessary to perform screening by amplifying a DNA fragment containing an entire region from a gene library using DNA fragments of the region as primers. When an entire region is amplified, the DNA fragment is subjected to agarose gel electrophoresis, followed by excision of an objective band. Thus the DNA fragment containing the AK gene can be recovered.

For DNA primers, single strand DNA's of 23 mer and 21 mer represented by 5'-TCGCGAAGTAGCACCTGTCACTT-3' (SEQ ID NO: 5 in Sequence Listing) and 5'-ACGGAATTCAATCTTACGGCC-3' (SEQ ID NO: 6 in Sequence Listing) are most suitable to amplify a region of about 1643 bp based on, for example, a sequence known for Corynebacterium glutamicum (see Molecular Microbiology (1991), 5(5), 1197-1204; Mol. Gen. Genet. (1990), 224, 317-324). The DNA can be synthesized in accordance with an ordinary method using a phosphoamidite method (see Tetrahedron Letters (1981), 22, 1859) by using a DNA synthesizer Model 380B produced by Applied Biosystems. The PCR can be performed by using DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd., using Taq DNA polymerase in accordance with a method designated by the supplier.

It is preferable for the mutant type AK gene amplified by the PCR method to be connected to vector DNA autonomously replicable in cells of E. coli and/or coryneform bacteria to prepare recombinant DNA which is introduced into E. coli cells, in order to facilitate following operations. The vector autonomously replicable in cells of E. coli is preferably a plasmid vector, preferably as those autonomously replicable in cells of a host, including, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, and RSF1010.

When these vectors are inserted with a DNA fragment having an ability to make the plasmid autonomously replicable in coryneform bacteria, they can be used as so-called shuttle vectors which are autonomously replicable in both of E. coli and coryneform bacteria. In order to introduce a plasmid into E. coli for transformation, it is possible to use, for example, a method of D. M. Morrison (Methods in Enzymology, 68, 326 (1979)), or a method for treating recipient cells with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)).

The wild type AK gene is obtained by isolating the AK gene from the AK wild strain as described above, and the mutant type AK gene is obtained by isolating the AK gene from the AK mutant strain.

The mutant type AK gene to be used for the present invention is not especially limited provided that it codes for AK

in which cumulative feedback inhibition by L-lysine and L-threonine is desensitized. However, its mutation may be exemplified, with respect to the amino acid sequence of the wild type AK, such that a 279th alanine residue from the N-terminal is changed to an amino acid residue other than alanine and other than acidic amino acids in α-subunit, and a 30th alanine residue is changed to an amino acid residue other than alanine and other than acidic amino acids in β-subunit. The amino acid sequence of the wild type AK is herein specifically exemplified by an amino acid sequence defined in SEQ ID NO: 10 in Sequence Listing for the α-subunit, and an amino acid sequence defined in SEQ ID NO: 12 in Sequence Listing for the β-subunit.

The aforementioned amino acid residue other than alanine and other than acidic amino acids is exemplified by threonine, arginine, cysteine, phenylalanine, proline, serine, tyrosine and valine residues.

Any codon corresponding to the amino acid residue to be replaced is available especially regardless of its type provided that it codes for the amino acid residue. It is postulated that the amino acid sequence of harbored wild type AK may slightly differ depending on difference in bacterial species and bacterial strains. AK having such replacement, deletion or insertion of amino acid residues at positions irrelevant to the activity of the enzyme can be also used for the present invention.

(3) Preparation of HD mutant strain and HD deficient strain

The HD mutant strain is obtained as described in (1) by performing a treatment of a coryneform bacterium which produces wild type HD with ultraviolet light irradiation or a mutating agent, and selecting a strain which produces mutant type HD from bacterial cells subjected to the mutation treatment. The HD mutant strain which expresses no wild type HD is also obtained by introducing a mutant type HD gene isolated from the HD mutant strain thus obtained into cells of a wild type coryneform bacterium, and performing gene replacement by way of homologous recombination with an HD gene on chromosome.

The mutant type HD gene may be replaced with the HD gene on the host chromosome as follows (see Fig. 1). Namely, a temperature-sensitive replication origin originating from Brevibacterium lactofermentum, the mutant type HD gene, and a marker gene for exhibiting resistance to a drug such as chloramphenicol are inserted into a plasmid vector to prepare recombinant DNA. The recombinant DNA is used to transform a coryneform bacterium, transformed strains are cultivated at a temperature at which the temperature-sensitive replication origin does not operate, and then they are cultivated in a medium containing the drug. Thus a transformed strain, in which the recombinant DNA is integrated into chromosomal DNA, is obtained.

The strain with the recombinant DNA integrated into the chromosome causes recombination with an HD gene sequence originally existing on the chromosome, in which two fused genes of the chromosomal HD gene and the mutant type HD gene are inserted into the chromosome in a state of interposing other portions of the recombinant DNA (vector portion, temperature-sensitive replication origin, and drug resistance marker). Therefore, the wild type HD is dominant in this state, and thus equivalent growth to that of the wild strain is exhibited in a minimum medium.

Next, in order to allow only the mutant type HD gene to remain on the chromosomal DNA, one copy of an HD gene is allowed to fall off together with the vector portion (including the temperature-sensitive replication origin and the drug resistance marker) by recombination of the two HD genes. For example, the strain with the integration on the chromosome is cultivated, and cultivated bacterial cells are spread and cultivated on a solid plate medium containing no drug. Grown colonies are replicated and cultivated on a solid plate medium containing the drug, and drug-sensitive strains are obtained. The fact that the vector portion falls off from chromosomes of the obtained drug-sensitive strains is confirmed by Southern hybridization, and the fact that the mutant type HD is expressed is confirmed.

When the gene replacement is performed by using an HD gene coding for a part of HD, that is a partially deleted HD gene, instead of the aforementioned mutant type HD gene, an HD deficient strain in which its chromosomal HD gene is replaced with the partially deleted HD gene is obtained.

As described in Example 1 below, it is postulated that a region on the N-terminal side in HD participates in the activity. Therefore, the site to be deleted in the HD gene is exemplified by a region on the N-terminal side, for example, a region within 350 amino acids, for example, a region of 100th to 200th amino acids or 250th to 350th amino acids from the N-terminal. Since the HD gene is located in the same operon as that of homoserine kinase existing downstream therefrom, it is preferable that the promoter site of the HD gene is not deleted so as not to inhibit expression of homoserine kinase.

Introduction of the recombinant DNA into cells of coryneform bacteria is possible by using a method in which recipient cells are treated with calcium chloride to increase permeability of DNA as reported for E. coli K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or a method in which introduction is performed in a growth stage so that cells can incorporate DNA (so-called competent cells) as reported for Bacillus subtilis (Duncan, C. H., Wilson, G. A. and Young, F. E., Gene, 1, 153 (1977)) . Alternatively, it is also possible to perform introduction into recipients for recombinant DNA after converting the DNA recipients into protoplasts or spheroplasts which easily incorporate recombinant DNA, as known for Bacillus subtilis, actinomycetes and yeasts (Chang, S. and Choen, S. N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G.

R., Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)).

In the protoplast method, a sufficiently high frequency can be obtained even by the method used for Bacillus subtilis described above. It is of course possible to utilize a method in which DNA is incorporated into protoplasts of the genus Corynebacterium or Brevibacterium in the presence of polyethylene glycol or polyvinyl alcohol and divalent metal ion as described in Japanese Patent Laid-open No. 57-183799. An equivalent result is obtained even by a method in which incorporation of DNA is facilitated by addition of carboxymethyl cellulose, dextran, Ficoll, Pluronic F68 (Serva Co.) instead of polyethylene glycol or polyvinyl alcohol.

Further, recombinant DNA can be introduced into recipients belonging to bacteria of the genus Brevibacterium or Corynebacterium by using an electric pulse method (Sugimoto et al., Japanese Patent Laid-open No. 2-207791).

The wild type coryneform bacteria into which the mutant type HD gene or the deletion type HD gene is introduced are exemplified by bacteria belonging to the genus Corynebacterium, bacteria belonging to the genus Brevibacterium having been hitherto classified into the genus Brevibacterium but united as bacteria belonging to the genus Corynebacterium at present, and bacteria belonging to the genus Brevibacterium closely relative to bacteria belonging to the genus Corynebacterium. Especially, glutamate-producing bacteria belonging to the genus Corynebacterium (Brevibacterium) are most preferable in the present invention. Examples of wild strains of the glutamate-producing bacteria belonging to the genus Corynebacterium (Brevibacterium) includes the followings. These wild strains as well as strains added with a property of L-lysine production to such strains can be used for the present invention in the same manner.

| | |
|---|---|
| Corynebacterium acetoacidophilum | ATCC 13870 |
| Corynebacterium acetoglutamicum | ATCC 15806 |
| Corynebacterium callunae | ATCC 15991 |
| Corynebacterium glutamicum | ATCC 13032 |
| | ATCC 13060 |
| Brevibacterium divaricatum | ATCC 14020 |
| Brevibacterium lactofermentum | ATCC 13869 |
| Corynebacterium lilium | ATCC 15990 |
| Corynebacterium melassecola | ATCC 17965 |
| Brevibacterium saccharolyticum | ATCC 14066 |
| Brevibacterium immariophilum | ATCC 14068 |
| Brevibacterium roseum | ATCC 13825 |
| Brevibacterium flavum | ATCC 13826 |
| Brevibacterium thiogenitalis | ATCC 19240 |
| Microbacterium ammoniaphilum | ATCC 15354 |

The coryneform bacteria which can be used for the present invention includes mutant strains having glutamate productivity or those lacking glutamate productivity, in addition to the wild strains having glutamate productivity as described above. At present, various artificial mutant strains of coryneform glutamate-producing bacteria are used as L-lysine-producing bacteria, and these strains can be also used for the present invention. Such artificial mutant strains include the followings: AEC (S-(2-aminoethyl)-cysteine) resistant mutant strains; mutant strains which require amino acid such as L-homoserine (Japanese Patent Publication Nos. 48-28078 and 56-6499); mutant strains which exhibit resistance to AEC and require amino acids such as L-leucine, L-homoserine, L-proline, L-serine, L-arginine, L-alanine, and L-valine (United States Patent Nos. 3708395 and 3825472); L-lysine-producing mutant strains which exhibit resistance to DL-$\alpha$-amino-$\varepsilon$-caprolactam, $\alpha$-amino-lauryllactam, aspartate-analog, sulfa drug, quinoid, N-lauroylleucine; L-lysine-producing mutant strains which exhibit resistance to inhibitors of oxyaloacetate decarboxylase or respiratory system enzymes (Japanese Patent Laid-open Nos. 50-53588, 50-31093, 52-102498, 53-9394, 53-86089, 55-9783, 55-9759, 56-32995 and 56-39778, and Japanese Patent Publication Nos. 53-43591 and 53-1833); L-lysine-producing mutant strains which require inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692); L-lysine-producing mutant strains which exhibit sensitivity to fluoropyruvic acid or temperature not less than 34°C (Japanese Patent Laid-open Nos. 55-9783 and 53-86090); and mutant strains of Brevibacterium or Corynebacterium which exhibit resistance to ethylene glycol and produce L-lysine (see United States Patent Application Serial No. 333455).

(4) Amplification of AK gene in HD mutant strain or HD deficient strain

AK undergoes feedback inhibition in the case of co-existence of L-lysine and L-threonine. However, coryneform bacteria which do not express wild type homoserine dehydrogenase cannot produce L-threonine, and thus AK does not undergo feedback inhibition.
Therefore, it is expected that the L-lysine productivity is improved if the AK gene is amplified in cells of coryneform bacteria which do not express wild type homoserine dehydrogenase. It is further expected that the L-lysine productivity is more improved if an inhibition-desensitized type AK gene is used as the AK gene to be amplified because feedback inhibition is more reduced.

The coryneform bacteria for introduction of the AK gene which do not express homoserine dehydrogenase are exemplified by the HD mutant strain or the HD deficient strain obtained as described in (3) above. However, the effect of improvement in L-lysine productivity is obtained owing to the amplification of the AK gene in the same manner even when an HD completely deficient strain obtained by a mutation treatment is used.

In order to amplify the AK gene or the mutant type AK gene in cells of such coryneform bacteria which do not express wild type homoserine dehydrogenase, the coryneform bacteria may be transformed with recombinant DNA comprising the AK gene or the mutant type AK gene and a vector autonomously replicable in cells of coryneform bacteria.

The vector used herein may be any one provided that it is autonomously replicable in cells of coryneform bacteria. Specifically, there may be exemplified pAJ655, pAJ1844, pAJ611, pAJ3148 and pAJ440 described above.

The method for transforming coryneform bacteria is exemplified by a method in which recipient cells are treated with calcium chloride to increase permeability of DNA as reported for E. coli K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or a method in which introduction is performed in a growth stage so that cells can incorporate DNA (so-called competent cells) as reported for Bacillus subtilis (Duncan, C. H., Wilson, G. A. and Young, F. E., Gene, 1, 153, (1977)). Alternatively, it is also possible to perform introduction into recipients for recombinant DNA after converting the DNA recipients into protoplasts or spheroplasts which easily incorporate recombinant DNA, as known for Bacillus subtilis, actinomycetes and yeasts (Chang, S. and Choen, S. N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)).

Additionally, the stability of recombinant DNA in a host can be improved by allowing the vector to harbor a marker gene such as drug resistance, or a gene to supplement auxotrophy of the host.

A promoter inherent to the AK gene may be used exactly as it is for expressing the AK gene or the mutant type AK gene. However, it is also available that a promoter of another gene which operates in coryneform bacteria is used to ligate it with a DNA sequence coding for AK or mutant type AK.

(5) Introduction of mutant type AK gene into chromosomal DNA of HD mutant strain or HD deficient strain

The L-lysine productivity can be improved by performing amplification of the AK gene in cells of the HD mutant strain or the HD deficient strain as described in (4) above. However, in order to increase the stability of the AK gene introduced into the HD mutant strain or the HD deficient strain, it is preferable to integrate the AK gene into chromosomal DNA. It is preferable herein to use a mutant type AK gene as the AK gene to be integrated into chromosomal DNA.

In order to integrate the mutant type AK gene into chromosomal DNA of a host, integration of the gene may be performed in the same manner as the mutant type HD gene or the deletion type HD gene. Namely, a temperature-sensitive replication origin originating from Brevibacterium lactofermentum, the mutant type AK gene, and a marker gene for providing resistance to a drug such as chloramphenicol are inserted into a plasmid vector to prepare recombinant DNA. The recombinant DNA is used to transform a coryneform bacterium, transformed strains are cultivated at a temperature at which the temperature-sensitive replication origin does not operate, and then they are cultivated in a medium containing the drug. Thus a transformed strain, in which the recombinant DNA is integrated into chromosomal DNA, is obtained.

The strain with the recombinant DNA integrated into the chromosomal DNA causes recombination with an AK gene sequence originally existing on the chromosome, in which two fused genes of the chromosomal AK gene and the mutant type AK gene are inserted into the chromosome in a state of interposing other portions of the recombinant DNA (vector portion, temperature-sensitive replication origin, and drug resistance marker). The mutant type AK is dominant in this state, and thus the phenotype is the mutant type. Therefore, the strain integrated with the gene may be used as it is. However, when approximately the same sequences are aligned in parallel on the chromosomal DNA, recombination may takes place again, and one of the AK genes is apt to fall off. Accordingly, it is preferable to obtain a gene-replaced strain in which only the mutant type AK gene remains on the chromosomal DNA. Namely, one copy of the AK gene is allowed to fall off together with the vector portion (including the temperature-sensitive replication origin and the drug resistance marker) by recombination of the two AK genes. For example, the strain with the integration on the chro-

mosome is cultivated, and cultivated bacterial cells are spread and cultivated on a solid plate medium containing no drug. Grown colonies are replicated and cultivated on a solid plate medium containing the drug, and drug-sensitive strains are obtained. The fact that the vector portion falls off from chromosomes of the obtained drug-sensitive strains is confirmed by Southern hybridization, and the fact that the mutant type AK is expressed is confirmed.

No problem arises even when the wild type AK gene remains on the chromosomal DNA in a complete form, as being different from the case of the gene replacement using the mutant type HD gene or the deletion type HD gene. Accordingly, the mutant type AK gene may be integrated at a site other than that for the AK gene on the chromosomal DNA.

(6) Production of L-lysine

L-lysine can be produced and accumulated in a culture by cultivating, in an appropriate medium, the HD mutant strain, the HD deficient strain, the strains of these types in which the AK gene is amplified, or the HD mutant strain or the HD deficient strain into which the mutant type AK gene is integrated.

The medium to be used includes an ordinary medium containing a carbon source, a nitrogen source, inorganic ions and optionally other organic components.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, or starch hydrolysate; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin $B_1$ and L-homoserine or yeast extract to be contained in appropriate amounts as organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

When the HD deficient strain is used, L-threonine and L-methionine, or L-homoserine is added to the medium in appropriate amount(s).

Cultivation is preferably carried out under an aerobic condition for 16-72 hours. The cultivation temperature is preferably controlled at 25°C to 37°C, and pH is preferably controlled at 5-7 during cultivation. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment. Collection of L-lysine from a cultivated liquor may be carried out by combining an ordinary ion exchange resin method, a precipitation method and other known methods.

Brief Description of the Drawings

Fig. 1 is a conceptual view of gene integration and gene replacement;

Fig. 2 is a view of comparison of amino acid sequences of HD genes of various microorganisms;

Fig. 3 is a view of comparison of amino acid sequences of HD genes of various microorganisms (continued);

Fig. 4 shows process of construction of p399AK9B and p399AKYB;

Fig. 5 shows L-lysine productivity and OD after cultivation of HD mutant strain and HD deficient strain;

Fig. 6 shows L-lysine productivity and OD after cultivation of HD mutant strain and HD deficient strain in which AK gene is amplified; and

Fig. 7 shows L-lysine productivity and OD after cultivation of HD mutant strain and HD deficient strain in which mutant type AK gene is integrated into chromosome.

Description of Preferred Embodiments

The present invention will be more concretely explained below with reference to Examples.

Example 1: Analysis of Wild Type HD Gene, Leaky Type HD Gene, and Inhibition-Desensitized Type HD Gene

Leaky type HD mutant strains and a mutant strain for producing HD with desensitized feedback inhibition by L-threonine were created from a wild strain of Brevibacterium lactofermentum. A wild type HD gene, leaky type HD genes and a inhibition-desensitized type HD gene were isolated from the wild and mutant strains, and analysis of their structures was performed. Brevibacterium lactofermentum AJ12036 strain (FERM BP-734) was used as the wild strain. Brevibacterium lactofermentum AJ12472 and AJ12937 strains were used as the leaky type HD mutant strains. Brevibacterium lactofermentum AI6080 strain was used as the inhibition-desensitized type HD mutant strain. These mutant strains were obtained as follows.

The AJ12036 strain is a strain obtained by deleting an originally existing plasmid, pAM330 from Brevibacterium lactofermentum 2256 strain (ATCC 13869), which produces wild type HD with respect to HD.

The AJ12472 and AJ12937 strains ar strains obtained from Brevibacterium lactofermentum 2256 strain (ATCC

13869) as a result of repeated breeding by mutation using L-lysine productivity as an index, which produce leaky type HD's. The AI6080 stain is a stain obtained from Brevibacterium lactofermentum 2256 strain (ATCC 13869) as a result of repeated breeding by mutation using L-threonine productivity as an index, which produc s inhibition-desensitized type HD.

(1) Amplification of HD gene by PCR method

A nucleotide sequence of the HD gene has been reported for Corynebacterium glutamicum (Peoples, O. P. et al, Molecular Microbiology, 2(1), 63-72 (1988)). It was speculated that the similarity of each HD gene sequence might be high between Brevibacterium lactofermentum and Corynebacterium glutamicum. Thus synthetic primer DNA's were prepared on the basis of the sequence of Corynebacterium glutamicum for use in the PCR method.

Chromosomal DNA's were prepared in accordance with an ordinary method from Brevibacterium lactofermentum AJ12036, AJ12472, AJ12937 and AI6080 strains. In order to amplify DNA fragments of about 1500 bp containing HD genes from these chromosomal DNA's, a DNA synthesizer Model 1381A (ABI Ltd.) was used to synthesize two species of primers of a 5' side primer H1 ((841)5'-CTGGG AAGGTGAATCGAATTT-3'(860), SEQ ID NO: 1 in Sequence Listing) and a 3' side primer H2 ((2410)5'-TCCGAGGTTTGCA GAAGATC-3'(2391), SEQ ID NO: 2 in Sequence Listing). Numbers in the parentheses indicate positions in the nucleotide sequence published by People et al. (Peoples, O. P. et al, Molecular Microbiology, 2(1), 63-72 (1988)). Obtained synthetic primers were purified by reversed phase HPLC.

The PCR was performed with a composition shown below using a PCR amplification apparatus (DNA Thermal Cycler PJ2000 produced by Takara Shuzo Co., Ltd.) and a PCR kit (Takara GeneAmp™ kit produced by Takara Shuzo Co., Ltd.).

Table 1

| Component | Concentration | Blended amount |
|---|---|---|
| Primer H1 | 0.25 μM | 25 pmol |
| Primer H2 | 0.25 μM | 25 pmol |
| dATP, dGTP, dTTP, dCTP | each 200 μM | 20 nmol |
| Taq DNA polymerase | 2.5 U/100 μL | 0.5 μL (5 U/μL) |
| Chromosomal DNA | | 1 μg |
| 10 x reaction buffer | | 10 μL |
| Water | | balance |
| | | (total amount: 100 μL) |

Conditions for denaturation of DNA, annealing of DNA, and polymerase reaction in the PCR were at 94°C for 1 minute, at 37°C for 2 minutes and at 75°C for 3 minutes respectively, and transition between each of the temperatures was performed for 1 second. DNA was amplified by repeating the reaction cycle by 25 cycles. As a result of confirmation of sizes of amplification reaction products thus obtained by using agarose gel electrophoresis, amplification of DNA fragment of about 1.4 Kbp was observed.

Thus the DNA fragments amplified from the chromosomal DNA of each of the AJ12036, AJ12472, AJ12937 and AI6080 strains were respectively cut with a restriction enzyme KpnI to obtain DNA fragments which were inserted into a KpnI site of a vector plasmid pHSG399 (see Takeshita, S. et al., Gene (1987), 61, 63-74) to obtain recombinant DNA's. The recombinant DNA's containing amplified fragments originating from the AJ12036, AJ12472, AJ12937 and AI6080 strains were designated as pHDW, pHDMI, pHDMII and pHDMIII, respectively. Each of the plasmids was introduced into E. coli JM109 strain to obtain transformants.

(2) Determination of nucleotide sequences of HD genes and analysis of mutation points

(1) Comparison of nucleotide sequences of wild and mutant type HD genes

Nucleotide sequences of the HD gene fragments of Brevibacterium lactofermentum AJ12036, AJ12472, AJ12937 and AI6080 strains obtained as described above were determined by the dideoxy method.

A determined nucleotide sequence of the wild type HD gene of the AJ12036 strain, and an amino acid sequence

deduced from the sequence are shown in SEQ ID NO: 3 in Sequence Listing. Further, the amino acid sequence is shown in SEQ ID NO: 4 in Sequence Listing. As a result of comparison of the sequence with the sequence of the HD gene of Corynebacterium glutamicum reported by Peoples et al. (Peoples, O. P. et al, Molecular Microbiology, 2(1), 63-72 (1988)), nucleotides were different at 4 places, and one of them was different at the amino acid level. The different points are shown below using the sequence of the HD gene of Corynebacterium glutamicum as a standard.

(1) $^{531}G \rightarrow C$ ($^{148}Gly \rightarrow {}^{148}Ala$)
(2) $^{1222}G \rightarrow C$
(3) $^{1318}G \rightarrow T$
(4) $^{1324}C \rightarrow G$

Such a diversity observed among HD genes of wild type strains of coryneform bacteria do not affect the HD activity, and the sequence of the HD gene of Corynebacterium glutamicum may be treated as equivalent of the sequence of the HD gene of Brevibacterium lactofermentum shown in SEQ ID NO: 3.

As a result of comparison of the nucleotide sequence of the wild type HD gene and the amino acid sequence deduced from the sequence of the AJ12036 strain with nucleotide sequences of the HD genes and amino acid sequences of the AJ12472, AJ12937 and AI6080 strains, mutation points were found for the AJ12472 strain at 2 places, for AJ12937 at 1 place, and for AI6080 at 1 place, all accompanying amino acid replacement. Further, it was found that exactly the same mutation was commonly present in the HD genes of the AJ12472 and AJ12937 strains at 1 place. Each of the mutation points is shown below.

Table 2

| Bacterial strain | Difference in nucleotide sequence | Mutation in amino acid residue |
|---|---|---|
| AJ12472 strain | $^{155}G \rightarrow T$ | $^{23}Leu \rightarrow Phe$ |
| | $^{398}G \rightarrow A$ | $^{104}Val \rightarrow Ile$ |
| AJ12937 strain | $^{398}G \rightarrow A$ | $^{104}Val \rightarrow Ile$ |
| AI6080 strain | $^{1266}C \rightarrow T$ | $^{393}Ser \rightarrow Phe$ |

Hereinafter, the mutation point of $^{155}G \rightarrow T$ ($^{23}Leu \rightarrow Phe$) is referred to as "mutation point 1", the mutation point of $^{398}G \rightarrow A$ ($^{104}Val \rightarrow Ile$) is referred to as "mutation point 2", and the mutation point of $^{1266}C \rightarrow T$ ($^{393}Ser \rightarrow Phe$) is referred to as "mutation point 3".

(2) Comparison of HD amino acid sequences and mutation points of Brevibacterium lactofermentum, Bacillus subtilis and E. coli

It is known that two kinds of HD genes (HD-1, HD-2) are present in E. coli, and any of them constitutes bifunctional enzyme with AK (Zakin, M. M. et al., J. B. C., 258, 3028-3031 (1983)). Further, a nucleotide sequence of an HD gene of Bacillus subtilis has been also determined (Parsot, C. and Cohen, G. N., J. B. C., 263(29), 14654-14660 (1988)). Comparison of these amino acid sequences with the amino acid sequence of the wild type HD of Brevibacterium lactofermentum is shown in Figs. 2 and 3.

According to the result, it is understood that most of sites having high homology are located in a region on the N-terminal side, and that sites having high homology are concentrated in a region having amino acid numbers of 100-230 especially in the amino acid sequence of HD of Brevibacterium lactofermentum. It is postulated that the active region of HD exists on the N-terminal side according to the aforementioned fact, the fact that the two mutation points of HD of Brevibacterium lactofermentum are located within about 100 amino acid residues from the N-terminal, especially the mutation point 1 is located at a position of 23 amino acid residues from the N-terminal, and the two mutation points are amino acid residues having high conservation with respect to HD-1 and HD-2 of E. coli, HD of Bacillus subtilis, and HD of Brevibacterium lactofermentum, and the fact that no sequence corresponding to about 100 amino acid residues on the C-terminal side of HD of Brevibacterium lactofermentum is present in HD-1 and HD-2 of E. coli.

On the other hand, nucleotide sequences of HD genes of Corynebacterium glutamicum with desensitized inhibition by L-threonine have been published. Namely, Sahm et al. have reported replacement of one 68th amino acid from the C-terminal due to point mutation (Reinscheid, D. J. et al., J. Bacteriol., 173(10), 3228-3230 (1991)), and Sinskey et al. have reported change in 17th amino acid and followings due to frame shift on account of point mutation, and deletion

of 7th amino acid and followings from the C-terminal (Archer, J. A. C. et al., Gene, 107, 53-59 (1991)). Further, the mutation of amino acid residue was at a position of 53th amino acid residue from the C-terminal in the inhibition-desensitized HD of the Brevibacterium lactofermentum AI6080 strain. Furthermore, the region on the C-terminal side which does not exist in HD-1 and HD-2 of E. coli exists in HD of Bacillus subtilis which undergoes feedback inhibition by L-threonine in the same manner as HD of Brevibacterium lactofermentum. Accordingly, it is speculated that the region of HD relating to the feedback inhibition by L-threonine exists on the C-terminal side.

Example 2: Preparation and Analysis of Wild Type AK Gene and Mutant Type AK Gene

(1) Construction of wild type and mutant type AK genes and plasmids containing them

Chromosomal DNA's were prepared in accordance with an ordinary method from Brevibacterium lactofermentum 2256 strain (ATCC 13869) and an L-lysine-producing mutant strain AJ3463 (FERM P-1987) obtained from the 2256 strain by a mutation treatment. AK genes were amplified from the chromosomal DNA's in accordance with the PCR method (polymerase chain reaction; see White, T. J. et al., Trends Genet., 5, 185 (1989)). DNA primers used in the amplification were based on a sequence known for Corynebacterium glutamicum (see Molecular Microbiology (1991), 5(5), 1197-1204, and Mol. Gen. Genet. (1990), 224, 317-324). In order to amplify a region of about 1643 bp coding for the AK gene, single strand DNA's of 23 mer and 21 mer having sequences of 5'-TCGCGAAGTAGCACCTGTCACTT-3' (SEQ ID NO: 5) and 5'-ACGGAATTCAATCTTACGGCC-3' (SEQ ID NO: 6) were synthesized. The DNA's were synthesized by using a DNA synthesizer Model 380B produced by Applied Biosystems, Ltd. in accordance with an ordinary method using the phosphoamidito method (see Tetrahedron Letters (1981), 22, 1859).

In the PCR, gene amplification was performed by using DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd., using Taq DNA polymerase in accordance with a method designated by the supplier. After confirming an amplified gene fragment of 1643 kb by agarose gel electrophoresis, the fragment excised from the gel was purified in accordance with an ordinary method, and cut with restriction enzymes NruI (produced by Takara Shuzo Co., Ltd.) and EcoRI (produced by Takara Shuzo Co., Ltd.).

pHSG399 (see Takeshita, S. et al., Gene (1987), 61, 63-74) was used for a vector for cloning the gene fragment. pHSG399 was cut with a restriction enzyme SmaI (produced by Takara Shuzo Co., Ltd.) and a restriction enzyme EcoRI, and ligated with the amplified AK gene fragment. The ligation of DNA was performed in accordance with a designated method by using a DNA ligation kit (produced by Takara Shuzo Co., Ltd.). Thus a plasmid was prepared in which pHSG399 is connected to the AK gene product amplified from the chromosome of Brevibacterium. A plasmid having the AK gene originating from the 2256 strain (ATCC 13869) as a wild strain was designated as p399AKY, and a plasmid having the AK gene originating from AJ3463 as an L-lysine-producing bacterium was designated as p399AK9.

A DNA fragment having an ability to enable plasmids to make autonomous replication in bacteria belonging to the genus Corynebacterium (hereinafter referred to as "Coryne.-ori") was introduced into p399AKY and p399AK9 respectively, to prepare plasmids carrying the AK genes autonomously replicable in bacteria belonging to the genus Corynebacterium. Coryne.-ori was prepared from a plasmid vector autonomously replicable in bacterial cells of both Escherichia coli and bacteria belonging to the genus Corynebacterium. Some of such plasmid vectors have been reported. However, in this case, a shuttle vector pHK4 was used which was prepared from a plasmid pAJ1844 autonomously replicable in cells of coryneform bacteria (see Japanese Patent Laid-open No. 58-216199) and a plasmid pHSG298 autonomously replicable in cells of Escherichia coli (see Takeshita, S. et al., Gene, 61, 63-74 (1987)).

The preparation method for pHK4 is described in detail in Japanese Patent Laid-open No. 5-7491, however, it may be outlined as follows. pAJ1844 was partially cut with a restriction enzyme Sau3AI, and ligated with pHSG298 completely cut with a restriction enzyme BamHI. DNA after the ligation was introduced into Brevibacterium lactofermentum AJ12036 (FERM-P7559). An electric pulse method (see Japanese Patent Laid-open No. 2-207791) was used as a method for transformation. Selection of transformants was performed by using M-CM2G plates containing 25 μg/ml of kanamycin (containing 5 g of glucose, 10 g of polypeptone, 10 g of yeast extract, 5 g of NaCl, 0.2 g of DL-methionine and 15 g of agar in 1 l of pure water (pH 7.2)). Plasmids were prepared from transformants, and one having the smallest size was selected and designated as pHK4. This plasmid can make autonomous replication in Escherichia coli and coryneform bacteria, and gives kanamycin resistance to a host.

pHK4 obtained as described above was cut with a restriction enzyme KpnI (produced by Takara Shuzo Co., Ltd.), and cut faces were blunt-ended. Formation of blunt ends was performed in accordance with a designated method by using a DNA Blunting kit (produced by Takara Shuzo Co., Ltd.). After the blunt end formation, a phosphatized BamHI linker (produced by Takara Shuzo Co., Ltd.) was connected, to make modification to allow a DNA fragment of the Coryne.-ori portion to be cut from pHK4 with only BamHI. This plasmid was cut with BamHI. A generated Coryne.-ori DNA fragment was ligated with p399AKY or p399AK9 having been cut with BamHI in the same manner, to prepare a plasmid autonomously replicable in bacteria belonging to the genus Corynebacterium and contained the AK gene.

A plasmid containing the wild type AK gene originating from p399AKY was designated as p399AKYB, and a plasmid containing the mutant type AK gene originating from p399AK9 was designated as p399AK9B. Process of construc-

tion of p399AK9B and p399AKYB is shown in Fig. 4. A strain AJ12691 obtained by introducing the mutant type AK plasmid p399AK9B into the AJ12036 strain (FERM-P7559) as a wild type strain of Brevibacterium lactofermentum has been deposited on April 10, 1992 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-12918, transferred to international deposition based on the Budapest Treaty on February 10, 1995, and deposited under a deposition number of FERM BP-4999.

(2) Determination of nucleotide sequences of wild type and mutant type AK genes of Brevibacterium lactofermentum

The plasmid p399AKY containing the wild type AK gene and the plasmid p399AK9 containing the mutant type AK gene were prepared from each of the transformants, and nucleotide sequences of the wild and mutant type AK genes were determined. The determination of the nucleotide sequences was performed in accordance with a method of Sanger (F. Sanger et al., Proc. Natl. Acad. Sci., 74, 5463 (1977) and so on).

The nucleotide sequence of the wild type AK gene encoded by p399AKY is shown in SEQ ID NO: 7 in Sequence Listing. On the other hand, the nucleotide sequence of the mutant type AK gene encoded by p399AK9 only had mutation of one base pair in which 1051th G was changed to A in SEQ ID NO: 7 as compared with the wild type AK. It is known for the AK gene that two subunits of α, β are encoded on an identical DNA strand in an identical reading frame (see Kalinowski, J. et al., Molecular Microbiology (1991), 5(5), 1197-1204). Judging from homology, it is speculated for the gene of this case that two subunits of α, β are encoded on an identical DNA strand in an identical reading frame.

An amino acid sequence of the α-subunit of the wild type AK protein deduced from the nucleotide sequence of DNA is shown in SEQ ID NO: 8 in Sequence Listing simultaneously with the DNA sequence. Only the amino acid sequence is shown in SEQ ID NO: 9. An amino acid sequence of the β-subunit of the wild type AK protein deduced from the nucleotide sequence of DNA is shown in SEQ ID NO: 10 in Sequence Listing simultaneously with the DNA sequence. Only the amino acid sequence is shown in SEQ ID NO: 11. Each of the subunits uses GTG as a start codon, and a corresponding amino acid is represented as methionine. However, this represents methionine, valine or formylmethionine.

On the other hand, the mutation on the mutant type AK gene sequence indicates occurrence of replacement of amino acid residues such that a 279th alanine residue is replaced with a threonine residue in the α-subunit, and a 30th alanine residue is replaced with a threonine residue in the β-subunit, with respect to the amino acid sequences (SEQ ID NOS: 8 and 10) of the wild type AK protein.

(3) AK activity of expression product of mutant type AK gene and evaluation of desensitization of inhibition

Strains were prepared in which the wild type AK plasmid p399AKYB and the mutant type AK plasmid p399AK9B were respectively introduced into the AJ12036 strain (FERM-P7559) as a wild type strain of Brevibacterium lactofermentum (Corynebacterium glutamicum). The gene introduction into Corynebacterium was performed in accordance with an electric pulse method. The AK activity was measured for Brevibacterium lactofermentum (Corynebacterium glutamicum) AJ12036 strain as the host, an AJ12690 strain harboring the wild type AK plasmid, and an AJ12691 (FERM-P12918) strain harboring the mutant type AK plasmid. The measurement of the activity was performed in accordance with an ordinary method (see Miyajima, R. et al., The Journal of Biochemistry (1968), 63(2), 139-148).

As shown in Table 3, it has been confirmed that owing to the introduction of the AK plasmids, the specific activity of AK is increased about 10-15 times, and that the cumulative inhibition by L-lysine and L-threonine is desensitized only for the strain with the introduced mutant type AK plasmid. Table 3 shows the AK specific activity and the degree of its cumulative inhibition by L-lysine and L-threonine with respect to solutions obtained by destroying bacterial cells of the wild type AJ12036 strain of Brevibacterium lactofermentum, the AJ12690 strain allowed to harbor the wild type AK plasmid, and the AJ12691 strain allowed to harbor the mutant type AK plasmid. L-lysine and L-threonine as inhibitors were added to give a final concentration of 1 mM, respectively.

Table 3

| Bacterial strain | AK specific activity (mU/mg protein) | |
|---|---|---|
| | No addition | +1mM L-lysine, +1mM L-threonine |
| AJ12036 | 19.0 | 2.6 |
| AJ12690 | 235.3 | 34.6 |
| AJ12691 | 210.5 | 145.3 |

(4) Improvement of mutant type AK gene by site-specific mutation

In order to further improve the mutant type AK obtained as described above, it was intended to replace the mutation point ($^{279}$Ala → Thr) of the mutant type AK with another amino acid residue by means of site-specific mutation. The method for site-specific mutation for causing desired mutation at a desired site includes, for example, a method using PCR (Higuchi, R., 61, in PCR Technology (Erlich, H. A. Eds., Stockton Press (1989))), a method using phage (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T. A. et al., Meth. in Enzymol., 154, 367 (1987)).

With respect to the species of amino acid residues to be introduced by mutation, 20 species of amino acids were classified in accordance with respective properties such as polarity and molecular structure, and representative 8 species (Arg, Asp, Cys, Phe, Pro, Ser, Tyr, Val) were selected. Amino acid mutation and nucleotide replacement at respective mutation points are shown in Table 4.

Table 4

| Identification of mutation | Mutation point and amino acid change |
|---|---|
| Thr | $^{279}$Ala GCT → Thr A*CT |
| Arg | $^{279}$Ala GCT → Arg C*G*T |
| Asp | $^{279}$Ala GCT → Asp GA*T |
| Cys | $^{279}$Ala GCT → Cys T*G*T |
| Phe | $^{279}$Ala GCT → Phe T*T*T |
| Pro | $^{279}$Ala GCT → Pro C*CT |
| Ser | $^{279}$Ala GCT → Ser T*CT |
| Tyr | $^{279}$Ala GCT → Tyr T*A*T |
| Val | $^{279}$Ala GCT → Val GT*T |

A method for introducing mutation used herein is as follows. Eight species of synthetic DNA of 23 mers, in which the codon for the 279th Ala residue for introducing mutation was replaced with codons for desired amino acid residues, were designed (5'-GCCAGGCGAG CGT GCCAAGGTTT-3': SEQ ID NO: 12 as synthetic DNA for introducing Arg; 5'-GCCAGGCGAG GAT GCCAAGGTTT-3': SEQ ID NO: 13 as synthetic DNA for introducing Asp; 5'-GCCAGGCGAG TGT GCCAAGGTTT-3': SEQ ID NO: 14 as synthetic DNA for introducing Cys; 5'-GCCAGGCGAG TTT GCCAAGGTTT-3': SEQ ID NO: 15 as synthetic DNA for introducing Phe; 5'-GCCAGGCGAG CCT GCCAAGGTTT-3': SEQ ID NO: 16 as synthetic DNA for introducing Pro; 5'-GCCAGGCGAG TCT GCCAAGGTTT-3': SEQ ID NO: 17 as synthetic DNA for introducing Ser; 5'-GCCAGGCGAG TAT GCCAAGGTTT-3': SEQ ID NO: 18 as synthetic DNA for introducing Tyr; and 5'-GCCAGGCGAG GTT GCCAAGGTTT-3': SEQ ID NO: 19 as synthetic DNA for introducing Val). Sixteen species of 23 mer single strand DNA's were synthesized together with their complementary sequences.

When an Arg residue is introduced, for example, the single strand DNA having the sequence 5'-GCCAGGCGAG CGT GCCAAGGTTT-3' (SEQ ID NO: 12), the single strand DNA as its complementary chain, the single strand DNA having the sequence of SEQ ID NO: 5, and the single strand DNA having the sequence of SEQ ID NO: 6 were used as primers, and the PCR method was performed using p399AKY as a template. In order to avoid introduction of nonspecific mutation, about 280 base pairs containing the mutation point were excised from prepared DNA with restriction enzymes (NaeI-AvaII), and replaced with a corresponding site of p399AKY to prepare a recombinant plasmid. The nucleotide sequence was confirmed for the replaced region.

Upon measurement and evaluation of the enzyme activities of the mutant type AK's harbored by each of 8 species of obtained recombinant plasmids, an AK completely deficient strain of E. coli, Gif106M1 was used as a host (Boy, E. and Patte, J. C., J. Bacteriol., 112, 84-92 (1972); Theze, J. et al., J. Bacteriol., 117, 133-143 (1974)), because no AK deficient strain was known for coryneform bacteria. Otherwise, AK of a host and AK from the plasmid may exist in a mixed manner, probably resulting in inaccurate measurement. Many genes of coryneform bacteria are known to be expressed in E. coli. Thus it was postulated that the AK gene could be expressed in Escherichia coli since it was linked downstream from a lac promoter on pHSG399.

E. coli Gif106M1 was transformed with the recombinant plasmids of the wild type and the eight species, cell-free

extracts were prepared from each of transformed strains, and analysis of enzyme was performed. The AK activity was measured in accordance with a method described in Miyajima, R. et al., The Journal of Biochemistry (1968), 63(2), 139-148. The degree of inhibition desensitization and the specific activity are shown in Table 5, in the case of addition of 5 mM of L-lysine, 5 mM of L-threonine, or each 2 mM of L-lysine and L-threonine.

Table 5

| | Specific activity (mU/mg protein) | 5 mM Lys (%) | 5 mM Thr (%) | 2 mM Lys + Thr (%) |
|---|---|---|---|---|
| AJ12036 | 5.6 | 52.0 | 87.0 | 7.0 |
| Wild type | 316.4 | 52.7 | 86.8 | 6.2 |
| Thr | 374.4 | 58.7 | 109.1 | 78.3 |
| Arg | 197.4 | 41.4 | 106.8 | 58.6 |
| Cys | 267.0 | 66.5 | 135.7 | 60.6 |
| Phe | 447.7 | 14.6 | 105.0 | 32.4 |
| Pro | 125.0 | 77.5 | 123.2 | 85.2 |
| Ser | 406.8 | 55.0 | 114.4 | 37.0 |
| Tyr | 425.6 | 16.1 | 104.8 | 32.2 |
| Val | 448.9 | 60.5 | 103.5 | 75.5 |

As a result, AK was inactivated in the case of change to acidic amino acid such as Asp, while the inhibition by L-lysine and L-threonine was desensitized in the case of change to any other amino acid.

Example 3: Evaluation of L-lysine Productivity of HD Mutant Strain and HD Deficient Strain

In order to compare effects on the L-lysine productivity exerted by the two kinds of mutant type HD and the HD deficiency, mutant type HD genes or an HD gene with a part of its sequence deleted was integrated into chromosome of an identical host to prepare gene-replaced strains each of which was used as HD mutant strains and an HD deficient strain, and evaluated for the L-lysine productivity.

(1) Preparation of plasmids for replacing mutant type HD genes and plasmid for replacing deficient type HD gene

Plasmids for gene replacement were prepared for introduction by homologous recombination of mutant type HD genes or an HD gene with a part of its sequence deleted, into chromosomal DNA of Brevibacterium lactofermentum AJ12036 strain (FERM BP-734) (obtained by deleting a cryptic plasmid, pAM330 from Brevibacterium lactofermentum 2256 strain (ATCC 13869)).

(1) Preparation of HD gene having mutation point 1

The mutant type HD genes obtained in Example 1 were two species including the mutant type HD gene (from AJ12472 strain) having the mutation point 1 ($^{155}$C → T ($^{23}$Leu → Phe)) and the mutation point 2 ($^{398}$G → A ($^{104}$Val → Ile)), and the mutant type gene (from AJ12937) having only the mutation point 2. In order to investigate the influence exerted by the mutation point 1 on the HD activity and the L-lysine productivity, a mutant type HD gene having only the mutation point 1 was prepared. Hereinafter, the mutant type HD having the mutation point 1 is referred to as HD-M1, the mutant type HD having the mutation point 2 is referred to as HD-M2, and the mutant type HD having both the mutation points 1 and 2 is referred to as HDM-12.

The plasmid pHDMI containing the HDM-12 gene was cut with a restriction enzyme TthIII1 for cutting between the both mutation points of 1 and 2, and KpnI for cutting a ligation point between the vector and the HD gene, to obtain a 5' side HD fragment having the mutation point 1. In the same manner, pHDW having the wild type HD gene was cut with TthIII1 and KpnI to obtain a 3' side HD fragment. The HD-M1 gene having only the mutation point 1 was obtained by ligating the 5' side HD fragment with the 3' side HD fragment thus obtained.

(2) Construction of plasmids for gene replacement

The HD-M1 gene having only the mutation point 1 obtained as described above was inserted into a KpnI site of a vector plasmid pHSG398 having a chloramphenicol resistance (Cm$^r$) gene. Further, a temperature-sensitive replication origin (TSori) originating from Brevibacterium lactofermentum wild strain was introduced into a BamHI site of pHSG398. Thus a plasmid pTSHDM1 for replacing the HD-M1 gene was constructed. TSori was prepared from a plasmid pHSC4 (see Japanese Patent Laid-open No. 5-7491) obtained by treating a plasmid pHK4 having Coryne.-ori with hydroxylamine in vitro, transforming Brevibacterium lactofermentum AJ12036 with plasmid DNA after the treatment, and recovering the plasmid from a transformed strain incapable of growth at a high temperature (34°C). Coryne.-ori can be excised from pHSC4 with BamHI and KpnI, however, the plasmid was modified to allow Coryne.-ori to be excised only by cutting with BamHI. pHSC4 was cut with a restriction enzyme KpnI (produced by Takara Shuzo Co., Ltd.), and cut faces were blunt-ended. Formation of blunt ends was performed in accordance with a designated method using a DNA Blunting kit (produced by Takara Shuzo Co., Ltd.). After the blunt end formation, a phosphatized BamHI linker (produced by Takara Shuzo Co., Ltd.) was connected, to make modification to allow a DNA fragment of the TSori portion to be excised from pHSC4 with only BamHI. Escherichia coli AJ12571 harboring pHSC4 has been deposited on October 11, 1990 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-11763, transferred to international deposition based on the Budapest Treaty on August 26, 1991, and deposited under a deposition number of FERM BP-3524.

Next, in the same manner, the plasmid pHDII having the HD-M2 gene having only the mutation point 2 was cut with KpnI to obtain an HD-M2 gene fragment which was inserted into a KpnI site of pHSG398. Subsequently, TSori was inserted into a BamHI site. Thus a plasmid pTSHDM2 for replacing the HD-M2 gene was constructed.

Further, the plasmid pHDMI having the HD-M12 gene having both the mutation points 1 and 2 was cut with KpnI to obtain an HD-M12 gene fragment which was inserted into a KpnI site of pHSG398. Subsequently, TSori was inserted into a BamHI site. Thus a plasmid pTSHDM12 for replacing the HD-M12 gene was constructed.

Further, the plasmid pHDW having the wild type HD gene was cut with AatII, and a portion between two AatII sites (nucleotide numbers of 716-722 and 1082-1087 in SEQ ID NO: 3) existing in the HD gene was deleted. Thus a plasmid containing an HD gene with its part deleted (HD-Δ gene) was prepared. This plasmid was cut with KpnI to obtain an HD-Δ gene fragment which was inserted into a KpnI site of pHSG398. Subsequently TSori was inserted into a BamHI site. Thus a plasmid pTSHDΔ for replacing the HD-Δ gene was constructed.

(3) Preparation of HD mutant strain and HD deficient strain

Transformation of Brevibacterium lactofermentum AJ12036 strain was performed in accordance with an electric pulse method (Sugimoto et al., Japanese Patent Laid-open No. 2-207791) by using the plasmids for replacing the mutant type HD genes pTSHDM1, pTSHDM2 and pTSHDM12, and the plasmid for replacing the deletion type HD gene pTSHDΔ obtained as described above.

Obtained transformed strains were cultivated by using an M-CM2G medium at 25°C until full growth (about 1-2 x $10^9$/ml) was achieved. Cultivated bacterial cells were diluted to give $10^5$ cells per one plate, spread on an M-CM2G solid plate medium containing chloramphenicol (5 μg/mL), and cultivated at 34°C for 2-7 days to obtain colonies. It was confirmed for the obtained colonies that no plasmid was contained in cells. Further, it was confirmed that the plasmids for gene replacement were integrated into chromosome by means of Southern hybridization analysis using linear pHSG398 as a probe.

In the strain of chromosomal integration obtained as described above, two fused genes of an HD gene originally existing on the chromosome and the mutant type or deletion type HD gene are inserted in a state of interposing the vector (including TSori).

Next, in order to leave only the mutant type HD gene or the deletion type HD gene on the chromosome, the wild type HD gene and the vector were allowed to fall off from the chromosomal DNA to obtain strains replaced with the mutant type HD genes and a strain replaced with the deletion type HD gene. The wild type HD gene and the vector were allowed to fall off as follows.

Each of the integrated strains was cultivated at 34°C in an M-CM2G medium containing chloramphenicol (10 μg/mL) until full growth (1-2 x $10^9$) was achieved. Cultivated bacterial cells were spread on an M-CM2G solid plate medium containing no chloramphenicol to give 50-200 colonies per one plate, and cultivated at 34°C. Grown colonies were replicated onto an M-CM2G solid plate medium containing chloramphenicol (5 μg/mL), and cultivated at 34°C to obtain chloramphenicol sensitive strains. It was confirmed by Southern hybridization that the vector fell off from chromosome of the obtained chloramphenicol sensitive strains. It was further confirmed that the mutant type HD or the deletion type HD was expressed. It was confirmed by nucleotide sequence determination of chromosomal DNA that the mutation points were introduced into the gene-replaced strains thus obtained.

The HD-M1 gene-replaced strain thus obtained is designated as HDM1 strain, the HD-M2 gene-replaced strain is designated as HDM2 strain, the HDM-12 gene-replaced strain is designated as HDM12 strain, and the HD-Δ gene-

replaced strain is designated as HDΔ strain.

(2) L-lysine productivity of HD mutant strain and HD deficient strain

The L-lysine productivity was investigated for the HDM1, HDM2 and HDM12 strains as the HD mutant strains, and for the HDΔ strain as the HD deficient strain. These HD mutant strains and HD deficient strain purified by single colony isolation, as well as the AJ12036 strain as the wild strain for HD were respectively cultivated in a flask of 500 mL added with 20 mL of an L-lysine production medium shown below at 31.5°C for 72 hours. The final OD ($OD_{562}$) and accumulated amount of L-lysine were examined.

(L-lysine production medium)

This medium was prepared by dissolving components described below (in 1 L), adjusting pH to 8.0 with KOH, sterilizing at 115°C for 15 minutes, and then adding 50 g/L of $CaCO_3$ having been sterilized by heat in a dry state.

| | |
|---|---|
| Glucose | 100 g |
| $(NH_4)_2SO_4$ | 55 g |
| $KH_2PO_4$ | 1 g |
| $MgSO_4 \cdot 7H_2O$ | 1 g |
| d-biotin | 500 μg |
| Thiamin-HCl | 2000 μg |
| $FeSO_4 \cdot 7H_2O$ | 0.01 g |
| $MnSO_4 \cdot 7H_2O$ | 0.01 g |
| Nicotinamide | 5 mg |
| Mamenou (T-N) | 1.05 g |
| GD113 | 0.05 ml |

Results are shown in Fig. 5. No remaining sugar was found in any strain. As clarified from the results, accumulation of L-lysine was scarcely observed in AJ12036 strain, while it was about 4 g/l for HDM1 strain, about 17 g/l for HDM2 strain, about 7.5 g/l for HDM12 strain, and about 30 g/l for HDΔ strain. Accumulation of L-lysine was observed in any of the latter strains. Especially the L-lysine productivity was remarkably improved in HDΔ strain. Further, it was clarified that L-lysine was accumulated by introduction of only the mutation point 1 into HD.

HDΔ strain did not grow in a minimum medium or another minimum medium added with L-threonine or L-methionine alone, however, its growth was recovered by addition of L-homoserine, or L-threonine and L-methionine. Any of HDM1, HDM2 and HDM12 strains could grow in a minimum medium containing neither L-threonine nor L-methionine.

Brevibacterium lactofermentum HDΔ strain was designated as Brevibacterium lactofermentum AJ12846. It has been deposited on March 1, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-14197, transferred to international deposition based on the Budapest Treaty on February 9, 1995, and deposited under a deposition number of FERM BP-4995.

Example 4: Effect of Amplification of AK Gene in HD Mutant Strains and HD Deficient Strain

As described in Example 3, it has been clarified that the L-lysine productivity is improved by introducing the mutant type HD genes and the deletion type HD gene into the wild strain. Further investigation was made for the effect expected by combining AK gene amplification with the mutant type HD genes and the deletion type HD gene.

It is known that AK undergoes concerted inhibition by L-lysine and L-threonine, however, the degree of inhibition is low in the case of the presence of each of them alone. Therefore, since no L-threonine is produced by the HD mutant strain and the HD deficient strain, it is expected that the L-lysine productivity may be also improved by amplification of the wild type AK gene. It is further expected that the L-lysine productivity may be more improved by introducing the gene coding for the mutant type AK obtained in Example 2 which does not undergo inhibition by L-lysine and L-threonine.

In order to investigate such a combined effect of introduction of the mutant type HD gene or the deletion type HD gene and amplification of the AK gene, plasmids containing the AK genes were introduced into the HD mutant strains

and the HD deficient strain obtained in Example 3, and the L-lysine productivity was evaluated.

Each of the strains of AJ12036 as a wild strain, HDM1, HDM2 and HDM12 as HD mutant strains, and HDΔ as an HD deficient strain was used as hosts respectively, and transformed with the plasmid (p399AKYB) having the wild type AK gene and Coryne.-ori and the plasmid (p399AK9B) having the mutant type AK. Namely, 5 species of the hosts were transformed with 2 species of the plasmids, and 10 species of transformed strains were obtained in total.

Two strains for each of the transformed strains of AJ12036, HDM1, HDM2, HDM12 and HDΔ were cultivated by using the aforementioned L-lysine production medium, and the L-lysine productivity was examined. However, the transformed strains harboring the p399AKYB plasmid and the p399AK9B plasmid were cultivated with addition of 10 μg/mL of chloramphenicol to both a medium used for pre-cultivation and the L-lysine production medium. The cultivation was performed with stirring at 31.5°C for 72 hours in a flask of 500 mL added with 20 mL of the medium.

According to the result as shown in Fig. 6, no improvement in L-lysine productivity was observed for the AJ12036 strain even when the wild type AK plasmid was introduced, while increase in accumulated amount of L-lysine was observed for the HD mutant strain and the HD deficient strain owing to the introduction of the wild type AK plasmid. Further, when the mutant type AK plasmid was introduced, the L-lysine productivity was more improved for any of the HD mutant strains and the HD deficient strain as compared with the case of the introduction of the wild type AK plasmid. Furthermore, accumulation of L-lysine in an amount of about 22 g/L was observed even in the case of the AJ12036 strain harboring the wild type HD gene owing to the introduction of the mutant type AK plasmid.

Example 5: Effect of Gene Replacement of Mutant Tyne AK Gene in HD Mutant Strains and HD Deficient Strain

(1)Creation of strains replaced with mutant type AK gene and mutant type HD genes, and strain replaced with mutant type AK and deletion type HD gene

The effect of combination of HD mutation and HD deficiency with AK gene amplification has been investigated in Example 4. This Example concerns strains created such that the mutant type HD gene or the deletion type HD gene is integrated on chromosome, and the mutant type AK gene is integrated on chromosome, in order to evaluate the L-lysine productivity.

A plasmid for gene replacement for integrating the mutant type AK gene into chromosomal DNA was obtained as follows.

A plasmid pAK9T for replacing the mutant type AK gene was constructed by inserting the temperature-sensitive replication origin (TSori) of Brevibacterium lactofermentum into a BamHI site existing at a vector portion of the plasmid p399AK9 obtained in Example 2 (plasmid with the mutant type AK gene fragment originating from Brevibacterium lactofermentum AJ3463 strain amplified from chromosome connected to pHSG399).

A strain introduced with the mutant type AK gene and the HD-M1 gene [($AK^{FBR}$+HDM1) strain] was obtained by integrating the mutant type AK gene by employing the plasmid for replacing the mutant type AK gene pAK9T, using the HDM1 strain obtained in Example 4 as a parent strain. pAK9T was introduced into the HDM1 strain by means of an electric pulse method (Sugimoto et al., Japanese Patent Laid-open No. 2-207791), and obtained transformed strains were cultivated at 25°C by using an M-CM2G medium until full growth (about 1-2 x $10^9$/ml) was achieved. Cultivated bacterial cells were diluted to give $10^5$ cells per one plate, spread on an M-CM2G solid plate medium containing chloramphenicol (5 μg/mL), and cultivated at 34°C for 2-7 days to obtain colonies. It was confirmed for obtained colonies that no plasmid was contained in cells. Further, integration of pAK9T into chromosome was confirmed by Southern hybridization analysis using linear pHSG399 as a probe.

In the strain of chromosomal integration obtained as described above, two fused genes of an AK gene originally existing on the chromosome and the mutant type AK gene are inserted into chromosome in a state of interposing the vector (including TSori). Next, in order to leave only the mutant type AK gene on chromosomal DNA, the wild type AK gene and the vector were allowed to fall off to obtain a strain replaced with the mutant type AK gene. The vector was allowed to fall off as follows.

The strain integrated with the mutant type AK gene was cultivated in an M-CM2G medium containing chloramphenicol (10 μg/mL) at 34°C until full growth (1-2 x $10^9$) was achieved. Cultivated bacterial cells were spread on an M-CM2G solid plate medium containing no chloramphenicol to give 50-200 colonies per one plate, and cultivated at 34°C. Among clones which formed colonies, a strain was selected in which the L-lysine productivity was improved as compared with the HDM1 strain as the parent strain.

In the same manner, a strain having the mutant type AK gene and the HD-M12 gene [($A^{FBR}$+HDM12) strain] was selected as a strain with improved L-lysine productivity compared with the HDM12 strain, by performing gene replacement employing pAK9T in the same manner as described above using the HDM12 strain as a parent strain.

On the other hand, a strain introduced with the mutant type AK gene and the HD-M2 gene [($AK^{FBR}$+HDM2) strain] was obtained by creating an $AK^{FBR}$ strain having the introduced mutant type AK gene as a parent strain by introducing pAK9T into Brevibacterium lactofermentum AJ12036 strain, followed by introduction of the HD-M2 gene. Namely, pAK9T was introduced into the AJ12036 strain by means of an electric pulse method (Sugimoto et al., Japanese Patent

Laid-open No. 2-207791), and obtained transformed strains were cultivated at 25°C by using an M-CM2G medium until full growth (about 1-2 x $10^9$/ml) was achieved. Cultivated bacterial cells were diluted to give $10^5$ cells per one plate, spread on an M-CM2G solid plate medium containing chloramphenicol (5 μg/mL), and cultivated at 34°C for 2-7 days to obtain colonies. It was confirmed for obtained colonies that no plasmid was contained in cells. Further, integration of pAK9T into chromosome was confirmed by Southern hybridization analysis using linear pHSG399 as a probe.

Next, a strain integrated with the mutant type AK gene was cultivated in an M-CM2G medium containing chloramphenicol (10 μg/mL) at 34°C until full growth (1-2 x $10^9$) was achieved. Cultivated bacterial cells were spread on an M-CM2G solid plate medium containing no chloramphenicol to give 50-200 colonies per one plate, and cultivated at 34°C. Grown colonies were replicated onto an M-CM2G solid plate medium containing chloramphenicol (5 μg/mL), and cultivated at 34°C to obtain a chloramphenicol sensitive strain. It was confirmed by Southern hybridization that the vector fell off from chromosome of the obtained chloramphenicol sensitive strain. It was further confirmed that the mutant type AK was expressed. It was confirmed by nucleotide sequence determination of chromosomal DNA that the gene-replaced strain thus obtained had the introduced mutation point.

The plasmid pTSHDM2 for replacing the HD-M2 gene was introduced into chromosome of the mutant type AK gene-replaced strain ($AK^{FBR}$ strain) thus obtained, by means of the electric pulse method in the same manner as described above, and a gene-replaced strain in which the plasmid fell off was obtained. The gene-replaced strain was selected in accordance with the improvement in L-lysine productivity and sensitivity to L-threonine or L-methionine.

In the same manner, a strain introduced with the mutant type AK gene and the HD-Δ gene [($AK^{FBR}$+HDΔ)] was obtained by using the mutant type AK gene-replaced strain ($AK^{FBR}$ strain) as a parent strain, performing gene replacement with pTSHDΔ in the same manner as described above, and selecting a clone auxotrophic for L-methionine and L-threonine due to HD deficiency.

For each of the gene-replaced strains obtained as described above, it was finally confirmed by nucleic acid sequence determination of DNA that mutation points were introduced. Further, it was confirmed by Southern hybridization that the plasmid fell off.

(2) Evaluation of L-lysine productivity of strains replaced with mutant type AK gene and mutant type HD genes, and strain replaced with mutant type AK gene and deletion type HD gene

The L-lysine productivity was evaluated for the four strains obtained as described above, namely, ($AK^{FBR}$+HDM1), ($AK^{FBR}$+HDM2), ($AK^{FBR}$+HDM12) and ($AK^{FBR}$+HDΔ) strains.

Each of these strains was cultivated at 31.5°C for 72 hours in a flask of 500 mL added with 20 mL of the aforementioned L-lysine production medium, to measure OD of culture liquid and the amount of accumulated L-lysine after the cultivation.

Results are shown in Fig. 7. The amount of produced L-lysine was about 19 g/L for the $AK^{FBR}$ strain, while it was about 21 g/L for the ($AK^{FBR}$+HDM1) strain, about 22 g/L for the ($AK^{FBR}$+HDM2) strain, about 20 g/L for the ($AK^{FBR}$+HDM12) strain, and about 35 g/L for the ($AK^{FBR}$+HDΔ) strain. It was indicated that the L-lysine productivity was more improved when the mutant type HD genes or the deletion type HD gene was introduced in combination with the mutant type AK gene than when they were introduced alone.

There was little difference in OD of media after completion of the cultivation between the $AK^{FBR}$ strain and any of the ($AK^{FBR}$+HDM1), ($AK^{FBR}$+HDM2) and ($AK^{FBR}$+HDM12) strains. However, OD was more decreased in the case of the ($AK^{FBR}$+HDΔ) strain as compared with the gene-replaced strain with the deletion type HD gene alone. No remaining sugar was found after completion of the cultivation in any of the strains.

The ($AK^{FBR}$+HDM2), ($AK^{FBR}$+HDM12) and ($AK^{FBR}$+HDΔ) strains were designated as AJ12848 (FERM P-14198), AJ12849 (FERM P-14199) and AJ12850 (FERM P-14200), respectively. They have been deposited on March 1, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under the deposition numbers described above in the parentheses respectively, transferred to international deposition based on the Budapest Treaty on February 9, 1995, and deposited under deposition numbers of FERM BP-4996, FERM BP-4997 and FERM BP-4998 respectively in this order.

Example 6: Measurement of Reverse Mutation Frequency of HD Completely Deficient Strain

The reverse mutation frequency for homoserine auxotrophy was compared between the completely deficient strains of HD (HDΔ and $AK^{FBR}$+HDΔ strains) obtained by the gene replacement on chromosome and an HD deficient strain ATCC 13287 obtained by treating living bacterial cells with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as an ordinary mutating treatment agent.

The method for comparison was as follows. Stocked bacterial strains were pre-cultivated in a nutrient-rich medium, and inoculated in an L-lysine production medium. After cultivation with stirring for 72 hours, the culture liquids were appropriately diluted. Colonies were formed on an M-CM2G solid plate medium, which were subsequently replicated onto a minimum medium for Brevibacterium containing neither L-methionine nor L-threonine. The ratio of colonies

grown on the minimum medium to those grown on the nutrient-rich medium was regarded as a ratio of reverse mutation. When no revertant strain could be observed by using this method, the number of bacterial cells was increased to be applied to the minimum medium, and the number of bacterial cells was changed in accordance with degrees of dilution to be applied to the nutrient-rich medium. The number of bacterial cells applied to the minimum medium was estimated, and the ratio was calculated.

Results of measurement of the reverse mutation frequencies of the aforementioned three strains by means of this method are shown in Table 6. Revertant strains considerably appeared upon completion of the cultivation in the case of the ATCC 13287 strain. On the contrary, no revertant strain was observed at all in the case of the HDΔ and $AK^{FBR}$+HDΔ strains prepared by chromosomal recombination. Further, the HDΔ strain with no occurrence of reverse mutation had a larger amount of produced L-lysine than the ATCC 13287 strain.

Table 6

| Bacterial strain | Reverse mutation frequency (%) | Accumulated amount of L-lysine (g/l) |
|---|---|---|
| ATCC 13287 | 40 | 20.0 |
| HDΔ | 0 | 30.0 |
| $AK^{FBR}$+HDΔ | 0 | 35.0 |

SEQENCE LISTING

```
INFORMATION FOR SEQ ID NO:1:
     SEQUENCE CHARACTERISTICS:
          LENGTH: 20
          TYPE: nucleic acid
          STRANDEDNESS: single
          TOPOLOGY: linear
     MOLECULAR TYPE: other..synthetic oligonucleotide
     SEQUENCE DESCRIPTION: SEQ ID NO:1:
CTGGGAAGGT GAATCGAATT

INFORMATION FOR SEQ ID NO:2:
     SEQUENCE CHARACTERISTICS:
          LENGTH: 20
          TYPE: nucleic acid
          STRANDEDNESS: single
          TOPOLOGY: linear
     MOLECULAR TYPE: other..synthetic oligonucleotide
     SEQUENCE DESCRIPTION: SEQ ID NO:2:
TCCGAGGTTT GCAGAAGATC 20

INFORMATION FOR SEQ ID NO:3:
     SEQUENCE CHARACTERISTICS:
          LENGTH: 1478
          TYPE: nucleic acid
          STRANDEDNESS: double
          TOPOLOGY: linear
     MOLECULAR TYPE: genomic DNA
     ORIGINAL SOURCE:
          ORGANISM: Brevibacterium lactofermentum
          STRAIN: AJ12036
     FEATURE:
          NAME/KEY: CDS
          LOCATION: 89..1423
          IDENTIFICATION METHOD: S
     SEQUENCE DESCRIPTION: SEQ ID NO:3:
GGTACCCTTT TTGTTTTGGA CACATGTAGG GTGGCCGAAA CAAAGTAATA GGACAACAAC      60
GCTCGACCGC GATTATTTTT GGAGAATC ATG ACC TCA GCA TCT GCC CCA AGC        112
                               Met Thr Ser Ala Ser Ala Pro Ser
                                1               5
TTT AAC CCC GGC AAG GGT CCC GGC TCA GCA GTC GGA ATT GCC CTT TTA       160
Phe Asn Pro Gly Lys Gly Pro Gly Ser Ala Val Gly Ile Ala Leu Leu
    10                  15                  20
GGA TTC GGA ACA GTC GGC ACT GAG GTG ATG CGT CTG ATG ACC GAG TAC       208
Gly Phe Gly Thr Val Gly Thr Glu Val Met Arg Leu Met Thr Glu Tyr
25                  30                  35                  40
GGT GAT GAA CTT GCG CAC CGC ATT GGT GGC CCA CTG GAG GTT CGT GGC       256
Gly Asp Glu Leu Ala His Arg Ile Gly Gly Pro Leu Glu Val Arg Gly
                45                  50                  55
ATT GCT GTT TCT GAT ATC TCA AAG CCA CGT GAA GGC GTT GCA CCT GAG       304
Ile Ala Val Ser Asp Ile Ser Lys Pro Arg Glu Gly Val Ala Pro Glu
            60                  65                  70
CTG CTC ACT GAG GAC GCT TTT GCA CTC ATC GAG CGC GAG GAT GTT GAC       352
Leu Leu Thr Glu Asp Ala Phe Ala Leu Ile Glu Arg Glu Asp Val Asp
        75                  80                  85
ATC GTC GTT GAG GTT ATC GGC GGC ATT GAG TAC CCA CGT GAG GTA GTT       400
Ile Val Val Glu Val Ile Gly Gly Ile Glu Tyr Pro Arg Glu Val Val
    90                  95                  100
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CTC GCA GCT CTG AAG GCC GGC AAG TCT GTT GTT ACC GCC AAT AAG GCT      448
Leu Ala Ala Leu Lys Ala Gly Lys Ser Val Val Thr Ala Asn Lys Ala
105                 110                 115                 120
CTT GTT GCA GCT CAC TCT GCT GAG CTT GCT GAT GCA GCG GAA GCC GCA      496
Leu Val Ala Ala His Ser Ala Glu Leu Ala Asp Ala Ala Glu Ala Ala
                125                 130                 135
AAC GTT GAC CTG TAC TTC GAG GCT GCT GTT GCA GCC GCA ATT CCA GTG      544
Asn Val Asp Leu Tyr Phe Glu Ala Ala Val Ala Ala Ala Ile Pro Val
            140                 145                 150
GTT GGC CCA CTG CGT CGC TCC CTG GCT GGC GAT CAG ATC CAG TCT GTG      592
Val Gly Pro Leu Arg Arg Ser Leu Ala Gly Asp Gln Ile Gln Ser Val
        155                 160                 165
ATG GGC ATC GTT AAC GGC ACC ACC AAC TTC ATC TTG GAC GCC ATG GAT      640
Met Gly Ile Val Asn Gly Thr Thr Asn Phe Ile Leu Asp Ala Met Asp
    170                 175                 180
TCC ACC GGC GCT GAC TAT GCA GAT TCT TTG GCT GAG GCA ACT CGT TTG      688
Ser Thr Gly Ala Asp Tyr Ala Asp Ser Leu Ala Glu Ala Thr Arg Leu
185                 190                 195                 200
GGT TAC GCC GAA GCT GAT CCA ACT GCA GAC GTC GAA GGC CAT GAC GCC      736
Gly Tyr Ala Glu Ala Asp Pro Thr Ala Asp Val Glu Gly His Asp Ala
                205                 210                 215
GCA TCC AAG GCT GCA ATT TTG GCA TCC ATC GCT TTC CAC ACC CGT GTT      784
Ala Ser Lys Ala Ala Ile Leu Ala Ser Ile Ala Phe His Thr Arg Val
            220                 225                 230
ACC GCG GAT GAT GTG TAC TGC GAA GGT ATC AGC AAC ATC AGC GCT GCC      832
Thr Ala Asp Asp Val Tyr Cys Glu Gly Ile Ser Asn Ile Ser Ala Ala
        235                 240                 245
GAC ATT GAG GCA GCA CAG CAG GCA GGC CAC ACC ATC AAG TTG TTG GCC      880
Asp Ile Glu Ala Ala Gln Gln Ala Gly His Thr Ile Lys Leu Leu Ala
    250                 255                 260
ATC TGT GAG AAG TTC ACC AAC AAG GAA GGA AAG TCG GCT ATT TCT GCT      928
Ile Cys Glu Lys Phe Thr Asn Lys Glu Gly Lys Ser Ala Ile Ser Ala
265                 270                 275                 280
CGC GTG CAC CCG ACT CTA TTA CCT GTG TCC CAC CCA CTG GCG TCG GTA      976
Arg Val His Pro Thr Leu Leu Pro Val Ser His Pro Leu Ala Ser Val
                285                 290                 295
AAC AAG TCC TTT AAT GCA ATC TTT GTT GAA GCA GAA GCA GCT GGT CGC     1024
Asn Lys Ser Phe Asn Ala Ile Phe Val Glu Ala Glu Ala Ala Gly Arg
            300                 305                 310
CTG ATG TTC TAC GGA AAC GGT GCA GGT GGC GCG CCA ACC GCG TCT GCT     1072
Leu Met Phe Tyr Gly Asn Gly Ala Gly Gly Ala Pro Thr Ala Ser Ala
        315                 320                 325
GTG CTT GGC GAC GTC GTT GGT GCC GCA CGA AAC AAG GTG CAC GGT GGC     1120
Val Leu Gly Asp Val Val Gly Ala Ala Arg Asn Lys Val His Gly Gly
    330                 335                 340
CGT GCT CCA GGT GAG TCC ACC TAC GCT AAC CTG CCG ATC GCT GAT TTC     1168
Arg Ala Pro Gly Glu Ser Thr Tyr Ala Asn Leu Pro Ile Ala Asp Phe
345                 350                 355                 360
GGT GAG ACC ACC ACT CGT TAC CAC CTC GAC ATG GAT GTG GAA GAT CGC     1216
Gly Glu Thr Thr Thr Arg Tyr His Leu Asp Met Asp Val Glu Asp Arg
                365                 370                 375
GTG GGC GTT TTG GCT GAA TTG GCT AGC CTG TTC TCT GAG CAA GGA ATC     1264
Val Gly Val Leu Ala Glu Leu Ala Ser Leu Phe Ser Glu Gln Gly Ile
            380                 385                 390
TCC CTG CGT ACA ATC CGA CAG GAA GAG CGC GAT GAT GAT GCA CGT CTG     1312
Ser Leu Arg Thr Ile Arg Gln Glu Glu Arg Asp Asp Asp Ala Arg Leu
        395                 400                 405
```

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ATC GTT GTC ACG CAC TCT GCG CTG GAA TCT GAT CTT TCC CGC ACC GTT       1360
Ile Val Val Thr His Ser Ala Leu Glu Ser Asp Leu Ser Arg Thr Val
    410                 415                 420
GAA CTG CTG AAG GCT AAG CCT GTT GTT AAG GCA ATC AAC AGT GTG ATC       1408
Glu Leu Leu Lys Ala Lys Pro Val Val Lys Ala Ile Asn Ser Val Ile
425                 430                 435                 440
CGC CTC GAA AGG GAC T AATTTTACTG ACATGGCAAT TGAACTGAAC GTCGGTCGTA    1464
Arg Leu Glu Arg Asp
                445
AGGTTACCGT CACG                                                       1478
```

INFORMATION FOR SEQ ID NO:4:
SEQUENCE CHARACTERISTICS:
LENGTH: 445
TYPE: amino acid
TOPOLOGY: linear
MOLECULAR TYPE: protein
SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Thr Ser Ala Ser Ala Pro Ser Phe Asn Pro Gly Lys Gly Pro Gly
1               5                   10                  15
Ser Ala Val Gly Ile Ala Leu Leu Gly Phe Gly Thr Val Gly Thr Glu
            20                  25                  30
Val Met Arg Leu Met Thr Glu Tyr Gly Asp Glu Leu Ala His Arg Ile
        35                  40                  45
Gly Gly Pro Leu Glu Val Arg Gly Ile Ala Val Ser Asp Ile Ser Lys
    50                  55                  60
Pro Arg Glu Gly Val Ala Pro Glu Leu Leu Thr Glu Asp Ala Phe Ala
65                  70                  75                  80
Leu Ile Glu Arg Glu Asp Val Asp Ile Val Val Glu Val Ile Gly Gly
                85                  90                  95
Ile Glu Tyr Pro Arg Glu Val Val Leu Ala Ala Leu Lys Ala Gly Lys
            100                 105                 110
Ser Val Val Thr Ala Asn Lys Ala Leu Val Ala Ala His Ser Ala Glu
        115                 120                 125
Leu Ala Asp Ala Ala Glu Ala Ala Asn Val Asp Leu Tyr Phe Glu Ala
    130                 135                 140
Ala Val Ala Ala Ala Ile Pro Val Val Gly Pro Leu Arg Arg Ser Leu
145                 150                 155                 160
Ala Gly Asp Gln Ile Gln Ser Val Met Gly Ile Val Asn Gly Thr Thr
                165                 170                 175
Asn Phe Ile Leu Asp Ala Met Asp Ser Thr Gly Ala Asp Tyr Ala Asp
            180                 185                 190
Ser Leu Ala Glu Ala Thr Arg Leu Gly Tyr Ala Glu Ala Asp Pro Thr
        195                 200                 205
Ala Asp Val Glu Gly His Asp Ala Ala Ser Lys Ala Ala Ile Leu Ala
    210                 215                 220
Ser Ile Ala Phe His Thr Arg Val Thr Ala Asp Asp Val Tyr Cys Glu
225                 230                 235                 240
Gly Ile Ser Asn Ile Ser Ala Ala Asp Ile Glu Ala Ala Gln Gln Ala
                245                 250                 255
Gly His Thr Ile Lys Leu Leu Ala Ile Cys Glu Lys Phe Thr Asn Lys
            260                 265                 270
Glu Gly Lys Ser Ala Ile Ser Ala Arg Val His Pro Thr Leu Leu Pro
        275                 280                 285
Val Ser His Pro Leu Ala Ser Val Asn Lys Ser Phe Asn Ala Ile Phe
    290                 295                 300
Val Glu Ala Glu Ala Ala Gly Arg Leu Met Phe Tyr Gly Asn Gly Ala
305                 310                 315                 320
```

```
Gly Gly Ala Pro Thr Ala Ser Ala Val Leu Gly Asp Val Val Gly Ala
                325                 330                 335
Ala Arg Asn Lys Val His Gly Gly Arg Ala Pro Gly Glu Ser Thr Tyr
            340                 345                 350
Ala Asn Leu Pro Ile Ala Asp Phe Gly Glu Thr Thr Thr Arg Tyr His
        355                 360                 365
Leu Asp Met Asp Val Glu Asp Arg Val Gly Val Leu Ala Glu Leu Ala
    370                 375                 380
Ser Leu Phe Ser Glu Gln Gly Ile Ser Leu Arg Thr Ile Arg Gln Glu
385                 390                 395                 400
Glu Arg Asp Asp Asp Ala Arg Leu Ile Val Val Thr His Ser Ala Leu
                405                 410                 415
Glu Ser Asp Leu Ser Arg Thr Val Glu Leu Leu Lys Ala Lys Pro Val
            420                 425                 430
Val Lys Ala Ile Asn Ser Val Ile Arg Leu Glu Arg Asp
        435                 440                 445
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INFORMATION FOR SEQ ID NO:5:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TCGCGAAGTA GCACCTGTCA CTT 23
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INFORMATION FOR SEQ ID NO:6:
SEQUENCE CHARACTERISTICS:
LENGTH: 21
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:6:

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ACGGAATTCA ATCTTACGGC C 21
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INFORMATION FOR SEQ ID NO:7:
SEQUENCE CHARACTERISTICS:
LENGTH: 1643
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULAR TYPE: genomic DNA
ORIGINAL SOURCE:
ORGANISM: Corynebacterium glutamicum
STRAIN: ATCC 13869
SEQUENCE DESCRIPTION: SEQ ID NO:7:

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TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC    60
TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT   120
GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG   180
GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAGGTGG CCCTGGTCGT ACAGAAATAT   240
GGCGGTTCCT CGCTTGAGAG TGCGGAACGC ATTAGAAACG TCGCTGAACG GATCGTTGCC   300
ACCAAGAAGG CTGGAAATGA TGTCGTGGTT GTCTGCTCCG CAATGGGAGA CACCACGGAT   360
GAACTTCTAG AACTTGCAGC GGCAGTGAAT CCCGTTCCGC CAGCTCGTGA AATGGATATG   420
CTCCTGACTG CTGGTGAGCG TATTTCTAAC GCTCTCGTCG CCATGGCTAT TGAGTCCCTT   480
GGCGCAGAAG CTCAATCTTT CACTGGCTCT CAGGCTGGTG TGCTCACCAC CGAGCGCCAC   540
GGAAACGCAC GCATTGTTGA CGTCACACCG GGTCGTGTGC GTGAAGCACT CGATGAGGGC   600
AAGATCTGCA TTGTTGCTGG TTTTCAGGGT GTTAATAAAG AAACCCGCGA TGTCACCACG   660
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TTGGGTCGTG GTGGTTCTGA CACCACTGCA GTTGCGTTGG CAGCTGCTTT GAACGCTGAT     720
GTGTGTGAGA TTTACTCGGA CGTTGACGGT GTGTATACCG CTGACCCGCG CATCGTTCCT     780
AATGCACAGA AGCTGGAAAA GCTCAGCTTC GAAGAAATGC TGGAACTTGC TGCTGTTGGC     840
TCCAAGATTT TGGTGCTGCG CAGTGTTGAA TACGCTCGTG CATTCAATGT GCCACTTCGC     900
GTACGCTCGT CTTATAGTAA TGATCCCGGC ACTTTGATTG CCGGCTCTAT GGAGGATATT     960
CCTGTGGAAG AAGCAGTCCT TACCGGTGTC GCAACCGACA AGTCCGAAGC CAAAGTAACC    1020
GTTCTGGGTA TTTCCGATAA GCCAGGCGAG GCTGCCAAGG TTTTCCGTGC GTTGGCTGAT    1080
GCAGAAATCA ACATTGACAT GGTTCTGCAG AACGTCTCCT CTGTGGAAGA CGGCACCACC    1140
GACATCACGT TCACCTGCCC TCGCGCTGAC GGACGCCGTG CGATGGAGAT CTTGAAGAAG    1200
CTTCAGGTTC AGGGCAACTG GACCAATGTG CTTTACGACG ACCAGGTCGG CAAAGTCTCC    1260
CTCGTGGGTG CTGGCATGAA GTCTCACCCA GGTGTTACCG CAGAGTTCAT GGAAGCTCTG    1320
CGCGATGTCA ACGTGAACAT CGAATTGATT TCCACCTCTG AGATCCGCAT TTCCGTGCTG    1380
ATCCGTGAAG ATGATCTGGA TGCTGCTGCA CGTGCATTGC ATGAGCAGTT CCAGCTGGGC    1440
GGCGAAGACG AAGCCGTCGT TTATGCAGGC ACCGGACGCT AAAGTTTTAA AGGAGTAGTT    1500
TTACAATGAC CACCATCGCA GTTGTTGGTG CAACCGGCCA GGTCGGCCAG GTTATGCGCA    1560
CCCTTTTGGA AGAGCGCAAT TTCCCAGCTG ACACTGTTCG TTTCTTTGCT TCCCCGCGTT    1620
CCGCAGGCCG TAAGATTGAA TTC                                            1643
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INFORMATION FOR SEQ ID NO:8:
     SEQUENCE CHARACTERISTICS:
          LENGTH: 1643
          TYPE: nucleic acid
          STRANDEDNESS: double
          TOPOLOGY: linear
     MOLECULAR TYPE: genomic DNA
     ORIGINAL SOURCE:
          ORGANISM: Corynebacterium glutamicum
          STRAIN: ATCC13869
     FEATURE:
          NAME/KEY: mat peptide
          LOCATION: 217..1479
          IDENTIFICATION METHOD: S
     SEQUENCE DESCRIPTION: SEQ ID NO:8:
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TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC      60
TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT     120
GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG     180
GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAG GTG GCC CTG GTC GTA CAG      234
                                        Met Ala Leu Val Val Gln
                                         1               5
AAA TAT GGC GGT TCC TCG CTT GAG AGT GCG GAA CGC ATT AGA AAC GTC      282
Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala Glu Arg Ile Arg Asn Val
            10                  15                  20
GCT GAA CGG ATC GTT GCC ACC AAG AAG GCT GGA AAT GAT GTC GTG GTT      330
Ala Glu Arg Ile Val Ala Thr Lys Lys Ala Gly Asn Asp Val Val Val
        25                  30                  35
GTC TGC TCC GCA ATG GGA GAC ACC ACG GAT GAA CTT CTA GAA CTT GCA      378
Val Cys Ser Ala Met Gly Asp Thr Thr Asp Glu Leu Leu Glu Leu Ala
    40                  45                  50
GCG GCA GTG AAT CCC GTT CCG CCA GCT CGT GAA ATG GAT ATG CTC CTG      426
Ala Ala Val Asn Pro Val Pro Pro Ala Arg Glu Met Asp Met Leu Leu
55                  60                  65                  70
ACT GCT GGT GAG CGT ATT TCT AAC GCT CTC GTC GCC ATG GCT ATT GAG      474
Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu Val Ala Met Ala Ile Glu
                75                  80                  85
TCC CTT GGC GCA GAA GCT CAA TCT TTC ACT GGC TCT CAG GCT GGT GTG      522
Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr Gly Ser Gln Ala Gly Val
            90                  95                  100
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CTC ACC ACC GAG CGC CAC GGA AAC GCA CGC ATT GTT GAC GTC ACA CCG        570
Leu Thr Thr Glu Arg His Gly Asn Ala Arg Ile Val Asp Val Thr Pro
        105                 110                 115
GGT CGT GTG CGT GAA GCA CTC GAT GAG GGC AAG ATC TGC ATT GTT GCT        618
Gly Arg Val Arg Glu Ala Leu Asp Glu Gly Lys Ile Cys Ile Val Ala
    120                 125                 130
GGT TTT CAG GGT GTT AAT AAA GAA ACC CGC GAT GTC ACC ACG TTG GGT        666
Gly Phe Gln Gly Val Asn Lys Glu Thr Arg Asp Val Thr Thr Leu Gly
135                 140                 145                 150
CGT GGT GGT TCT GAC ACC ACT GCA GTT GCG TTG GCA GCT GCT TTG AAC        714
Arg Gly Gly Ser Asp Thr Thr Ala Val Ala Leu Ala Ala Ala Leu Asn
                155                 160                 165
GCT GAT GTG TGT GAG ATT TAC TCG GAC GTT GAC GGT GTG TAT ACC GCT        762
Ala Asp Val Cys Glu Ile Tyr Ser Asp Val Asp Gly Val Tyr Thr Ala
            170                 175                 180
GAC CCG CGC ATC GTT CCT AAT GCA CAG AAG CTG GAA AAG CTC AGC TTC        810
Asp Pro Arg Ile Val Pro Asn Ala Gln Lys Leu Glu Lys Leu Ser Phe
        185                 190                 195
GAA GAA ATG CTG GAA CTT GCT GCT GTT GGC TCC AAG ATT TTG GTG CTG        858
Glu Glu Met Leu Glu Leu Ala Ala Val Gly Ser Lys Ile Leu Val Leu
    200                 205                 210
CGC AGT GTT GAA TAC GCT CGT GCA TTC AAT GTG CCA CTT CGC GTA CGC        906
Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn Val Pro Leu Arg Val Arg
215                 220                 225                 230
TCG TCT TAT AGT AAT GAT CCC GGC ACT TTG ATT GCC GGC TCT ATG GAG        954
Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu Ile Ala Gly Ser Met Glu
                235                 240                 245
GAT ATT CCT GTG GAA GAA GCA GTC CTT ACC GGT GTC GCA ACC GAC AAG       1002
Asp Ile Pro Val Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys
            250                 255                 260
TCC GAA GCC AAA GTA ACC GTT CTG GGT ATT TCC GAT AAG CCA GGC GAG       1050
Ser Glu Ala Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu
        265                 270                 275
GCT GCC AAG GTT TTC CGT GCG TTG GCT GAT GCA GAA ATC AAC ATT GAC       1098
Ala Ala Lys Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp
    280                 285                 290
ATG GTT CTG CAG AAC GTC TCC TCT GTG GAA GAC GGC ACC ACC GAC ATC       1146
Met Val Leu Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile
295                 300                 305                 310
ACG TTC ACC TGC CCT CGC GCT GAC GGA CGC CGT GCG ATG GAG ATC TTG       1194
Thr Phe Thr Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu
                315                 320                 325
AAG AAG CTT CAG GTT CAG GGC AAC TGG ACC AAT GTG CTT TAC GAC GAC       1242
Lys Lys Leu Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp
            330                 335                 340
CAG GTC GGC AAA GTC TCC CTC GTG GGT GCT GGC ATG AAG TCT CAC CCA       1290
Gln Val Gly Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro
        345                 350                 355
GGT GTT ACC GCA GAG TTC ATG GAA GCT CTG CGC GAT GTC AAC GTG AAC       1338
Gly Val Thr Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn
    360                 365                 370
ATC GAA TTG ATT TCC ACC TCT GAG ATC CGC ATT TCC GTG CTG ATC CGT       1386
Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg
375                 380                 385                 390
GAA GAT GAT CTG GAT GCT GCT GCA CGT GCA TTG CAT GAG CAG TTC CAG       1434
Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln
                395                 400                 405
```

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CTG GGC GGC GAA GAC GAA GCC GTC GTT TAT GCA GGC ACC GGA CGC TAA      1482
Leu Gly Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
        410                 415                 420 421
AGTTTTAAAG GAGTAGTTTT ACAATGACCA CCATCGCAGT TGTTGGTGCA ACCGGCCAGG   1542
TCGGCCAGGT TATGCGCACC CTTTTGGAAG AGCGCAATTT CCCAGCTGAC ACTGTTCGTT   1602
TCTTTGCTTC CCCGCGTTCC GCAGGCCGTA AGATTGAATT C                       1643
```

```
INFORMATION FOR SEQ ID NO:9:
     SEQUENCE CHARACTERISTICS:
           LENGTH: 421
           TYPE: amino acid
           TOPOLOGY: linear
     MOLECULAR TYPE: protein
     SEQUENCE DESCRIPTION: SEQ ID NO:9:
     Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
     1               5                   10                  15
     Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
                 20                  25                  30
     Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
             35                  40                  45
     Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
         50                  55                  60
     Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
     65                  70                  75                  80
     Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                     85                  90                  95
     Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
                 100                 105                 110
     Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
             115                 120                 125
     Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
         130                 135                 140
     Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
     145                 150                 155                 160
     Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
                     165                 170                 175
     Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
                 180                 185                 190
     Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
             195                 200                 205
     Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
         210                 215                 220
     Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
     225                 230                 235                 240
     Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
                     245                 250                 255
     Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
                 260                 265                 270
     Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
             275                 280                 285
     Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
         290                 295                 300
     Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ala Asp Gly Arg
     305                 310                 315                 320
     Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
                     325                 330                 335
     Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
                 340                 345                 350
```

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Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
        355                 360                 365
Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
    370                 375                 380
Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385                 390                 395                 400
Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
                405                 410                 415
Ala Gly Thr Gly Arg
            420
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INFORMATION FOR SEQ ID NO:10:
SEQUENCE CHARACTERISTICS:
LENGTH: 1643
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULAR TYPE: genomic DNA
ORIGINAL SOURCE:
ORGANISM: Corynebacterium glutamicum
STRAIN: ATCC13869
FEATURE:
NAME/KEY: mat peptide
LOCATION: 964..1479
IDENTIFICATION METHOD: S
SEQUENCE DESCRIPTION: SEQ ID NO:10:

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TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC    60
TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT   120
GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG   180
GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAGGTGG CCCTGGTCGT ACAGAAATAT   240
GGCGGTTCCT CGCTTGAGAG TGCGGAACGC ATTAGAAACG TCGCTGAACG GATCGTTGCC   300
ACCAAGAAGG CTGGAAATGA TGTCGTGGTT GTCTGCTCCG CAATGGGAGA CACCACGGAT   360
GAACTTCTAG AACTTGCAGC GGCAGTGAAT CCCGTTCCGC CAGCTCGTGA AATGGATATG   420
CTCCTGACTG CTGGTGAGCG TATTTCTAAC GCTCTCGTCG CCATGGCTAT TGAGTCCCTT   480
GGCGCAGAAG CTCAATCTTT CACTGGCTCT CAGGCTGGTG TGCTCACCAC CGAGCGCCAC   540
GGAAACGCAC GCATTGTTGA CGTCACACCG GGTCGTGTGC GTGAAGCACT CGATGAGGGC   600
AAGATCTGCA TTGTTGCTGG TTTTCAGGGT GTTAATAAAG AAACCCGCGA TGTCACCACG   660
TTGGGTCGTG GTGGTTCTGA CACCACTGCA GTTGCGTTGG CAGCTGCTTT GAACGCTGAT   720
GTGTGTGAGA TTTACTCGGA CGTTGACGGT GTGTATACCG CTGACCCGCG CATCGTTCCT   780
AATGCACAGA AGCTGGAAAA GCTCAGCTTC GAAGAAATGC TGGAACTTGC TGCTGTTGGC   840
TCCAAGATTT TGGTGCTGCG CAGTGTTGAA TACGCTCGTG CATTCAATGT GCCACTTCGC   900
GTACGCTCGT CTTATAGTAA TGATCCCGGC ACTTTGATTG CCGGCTCTAT GGAGGATATT   960
CCT GTG GAA GAA GCA GTC CTT ACC GGT GTC GCA ACC GAC AAG TCC GAA    1008
    Met Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu
     1               5                  10                  15
GCC AAA GTA ACC GTT CTG GGT ATT TCC GAT AAG CCA GGC GAG GCT GCC    1056
Ala Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala
                20                  25                  30
AAG GTT TTC CGT GCG TTG GCT GAT GCA GAA ATC AAC ATT GAC ATG GTT    1104
Lys Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val
            35                  40                  45
CTG CAG AAC GTC TCC TCT GTG GAA GAC GGC ACC ACC GAC ATC ACG TTC    1152
Leu Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe
        50                  55                  60
ACC TGC CCT CGC GCT GAC GGA CGC CGT GCG ATG GAG ATC TTG AAG AAG    1200
Thr Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys
    65                  70                  75
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CTT CAG GTT CAG GGC AAC TGG ACC AAT GTG CTT TAC GAC GAC CAG GTC      1248
Leu Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val
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GGC AAA GTC TCC CTC GTG GGT GCT GGC ATG AAG TCT CAC CCA GGT GTT      1296
Gly Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val
                100                 105                 110
ACC GCA GAG TTC ATG GAA GCT CTG CGC GAT GTC AAC GTG AAC ATC GAA      1344
Thr Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu
            115                 120                 125
TTG ATT TCC ACC TCT GAG ATC CGC ATT TCC GTG CTG ATC CGT GAA GAT      1392
Leu Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp
        130                 135                 140
GAT CTG GAT GCT GCT GCA CGT GCA TTG CAT GAG CAG TTC CAG CTG GGC      1440
Asp Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly
    145                 150                 155
GGC GAA GAC GAA GCC GTC GTT TAT GCA GGC ACC GGA CGC TAAAGTTTAA       1490
Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
160                 165                 170         172
AGGAGTAGTT TTACAATGAC CACCATCGCA GTTGTTGGTG CAACCGGCCA GGTCGGCCAG    1550
GTTATGCGCA CCCTTTTGGA AGAGCGCAAT TTCCCAGCTG ACACTGTTCG TTTCTTTGCT    1610
TCCCCGCGTT CCGCAGGCCG TAAGATTGAA TTC                                 1643
```

INFORMATION FOR SEQ ID NO:11:

SEQUENCE CHARACTERISTICS:

LENGTH: 172

TYPE: amino acid

TOPOLOGY: linear

MOLECULAR TYPE: protein

SEQUENCE DESCRIPTION: SEQ ID NO:11:

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Met Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu Ala
1               5                   10                  15
Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala Lys
            20                  25                  30
Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val Leu
        35                  40                  45
Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe Thr
    50                  55                  60
Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys Leu
65                  70                  75                  80
Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val Gly
                85                  90                  95
Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val Thr
            100                 105                 110
Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu Leu
        115                 120                 125
Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp Asp
    130                 135                 140
Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly Gly
145                 150                 155                 160
Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
                165                 170
```

INFORMATION FOR SEQ ID NO:12:

SEQUENCE CHARACTERISTICS:

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other..synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:12:
GCCAGGCGAG CGTGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:13:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:13:
GCCAGGCGAG GATGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:14:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:14:
GCCAGGCGAG TGTGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:15:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:15:
GCCAGGCGAG TTTGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:16:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:16:
GCCAGGCGAG CCTGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:17:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other..synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID NO:17:
GCCAGGCGAG TCTGCCAAGG TTT 23

INFORMATION FOR SEQ ID NO:18:
SEQUENCE CHARACTERISTICS:
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

```
        MOLECULAR TYPE: other..synthetic oligonucleotide
        SEQUENCE DESCRIPTION: SEQ ID NO:18:
   GCCAGGCGAG TATGCCAAGG TTT 23

   INFORMATION FOR SEQ ID NO:19:
        SEQUENCE CHARACTERISTICS:
             LENGTH: 23
             TYPE: nucleic acid
             STRANDEDNESS: single
             TOPOLOGY: linear
        MOLECULAR TYPE: other..synthetic oligonucleotide
        SEQUENCE DESCRIPTION: SEQ ID NO:19:
   GCCAGGCGAG GTTGCCAAGG TTT 23
```

## Claims

1. A DNA fragment which codes for homoserine dehydrogenase originating from a coryneform bacterium in which at least one of a 23rd leucine residue and a 104th valine residue as counted from its N-terminus is changed to another amino acid residue.

2. A coryneform bacterium which harbors a gene coding for mutant type homoserine dehydrogenase originating from a coryneform bacterium in which at least one of a 23rd leucine residue and a 104th valine residue as counted from its N-terminus is changed to another amino acid residue.

3. The coryneform bacterium according to claim 2, which is transformed by integrating said gene coding for mutant type homoserine dehydrogenase into chromosomal DNA by way of homologous recombination with a homoserine dehydrogenase gene on a chromosome of the coryneform bacterium.

4. The coryneform bacterium according to claim 2 or 3, wherein said other amino acid residue is a phenylalanine residue for the 23rd leucine residue, and an isoleucine residue for the 104th valine residue.

5. A coryneform bacterium wherein its homoserine dehydrogenase gene is destroyed by integrating a DNA fragment coding for a part of homoserine dehydrogenase originating from a coryneform bacterium into chromosomal DNA by way of homologous recombination with a homoserine dehydrogenase gene on a chromosome of the coryneform bacterium.

6. A coryneform bacterium which harbors in its cells recombinant DNA constructed by ligating an aspartokinase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of coryneform bacteria, and expresses no wild type homoserine dehydrogenase.

7. The coryneform bacterium according to claim 6, wherein said aspartokinase gene is a gene coding for aspartokinase in which feedback inhibition by L-lysine and L-threonine is substantially desensitized.

8. A coryneform bacterium which is transformed by integrating, into chromosomal DNA of the coryneform bacterium, a gene coding for aspartokinase originating from a coryneform bacterium with desensitized feedback inhibition by L-lysine and L-threonine, and expresses no wild type homoserine dehydrogenase.

9. The coryneform bacterium according to claim 7 or 8, wherein the aspartokinase with desensitized feedback inhibition by L-lysine and L-threonine is mutant type aspartokinase in which a 279th alanine residue from the N-terminal is changed to an amino acid residue other than alanine and other than acidic amino acid in its $\alpha$-subunit, and a 30th alanine residue is changed to an amino acid residue other than alanine and other than acidic amino acid in its $\beta$-subunit.

10. The coryneform bacterium according to any one of claims 6-9, which is transformed by integrating, into its chromosome, a mutant type homoserine dehydrogenase gene for homoserine dehydrogenase originating from a coryneform bacterium in which at least one of a 23rd leucine residue and a 104th valine residue as counted from the N-

terminal is changed to another amino acid residue, by way of homologous recombination with a homoserine dehydrogenase gene on the chromosome of the coryneform bacterium, and thus expresses no wild type homoserine dehydrogenase.

11. The coryneform bacterium according to any one of claims 6-9, which has its homoserine dehydrogenase gene destroyed by integrating, into its chromosome, a DNA fragment coding for a part of homoserine dehydrogenase originating from a coryneform bacterium, by way of homologous recombination with a homoserine dehydrogenase gene on the chromosome of the coryneform bacterium, and expresses no wild type homoserine dehydrogenase.

12. A method of producing L-lysine comprising the steps of cultivating the coryneform bacterium according to any one of claims 2-11 in an appropriate medium, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture.

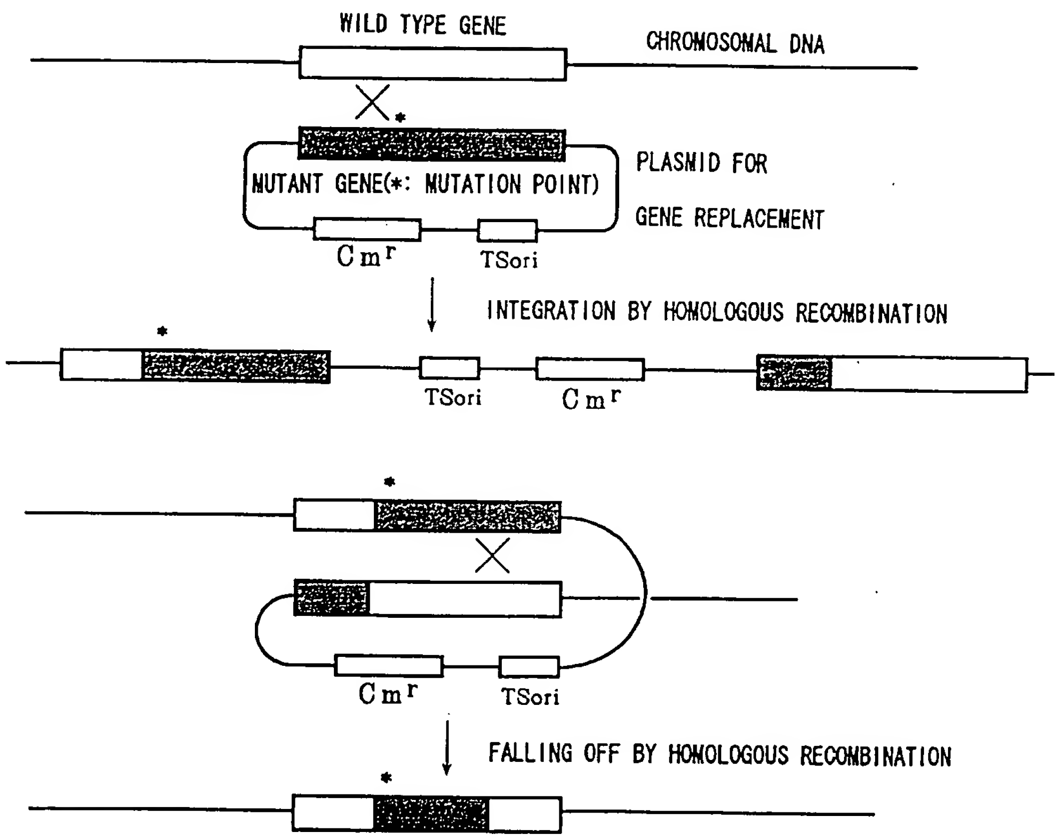
WILD TYPE GENE
CHROMOSOMAL DNA
MUTANT GENE(*: MUTATION POINT)
PLASMID FOR
GENE REPLACEMENT
Cm$^r$
TSori
INTEGRATION BY HOMOLOGOUS RECOMBINATION
TSori
Cm$^r$
Cm$^r$
TSori
FALLING OFF BY HOMOLOGOUS RECOMBINATION

Fig. 1

```
BL   M T S A S A P S F N P G K G P G S A V G I A L L G F G T V G T E   32
BS                         M K - - - - A I R V G L L G L G T V G S G   17
E1   V R V T H Q M L F N T D Q - - - - V I E V F V I G V G G V G G A   479
E2   I Q G L H Q S V F R A E K - - - - R I G L V L F G K G N I G S R   471

BL   V M R L M T E Y G D E L A H R I G G P L E V R G I A - V S D I S   63
BS   V V K I I Q D H Q D K L M H Q V G C P - - V - T I K K V - - - -   42
E1   L L E Q L K R Q Q S W L K N K - - - H I D L R V C - G V A N - S   506
E2   W L E L F A R E Q S T L S A R - - - T G F E F V L A G V V D - S   499

BL   K - - - - - - P R E G V A P E L L T E D A F A L I E R E D V D I V   90
BS   - - - - - - - - - - - - - - L V K D L E K K R E V D L P K E V L T T   62
E1   K - A L L T E V H G L N L E N W Q E E L A Q A K E P F N L G R L   537
E2   R R S L L S Y - D G L D A S R A L A F F N D E A V E Q D E E S L   530

BL   V E V I G G I E Y P R E - V V L A A - - - - - - - - - - - - - - L   108
BS   E - V Y D V I D D P D V D V V I E V I G G V E Q T K Q Y L V D A   93
E1   I R L V K E Y H L L N - P V I V N C T S S Q A V A D Q Y A D F L   568
E2   F - L W M R A H P Y D D L V V L D V T A S Q Q L A D Q Y L D F A   561

BL   - K A G K S V V T A N K - A L V A A H S A E - - L A D A A E A A   136
BS   L R S K K H V V T A N K D L M A - - - - V Y G S E L L A E A K E   121
E1   - R E G F H V V T P N K K A N T S S M D Y Y H Q L R Y A A E K S   599
E2   - S H G F H V I S A N K L A G A S D S N K Y R Q I H D A F E K T   592

BL   N V D - L Y F E A A V A A A I P V V G P L R R S L - A G D Q I Q   166
BS   N G C D I Y F E A S V A G G I P I L R T L E E G L S S - D R I T   152
E1   R R K F L Y - D I N V G A G L P V I E N L Q N L L N A G D E L M   630
E2   G R H W L Y - N A T V G A G L P I N H T V R D L I D S G D T I L   623

BL   S V M G I V N G T T N F I L D A M - D S T G A D Y A D S L A E A   197
BS   K M M G I V N G T T N F I L T K M I K E K S P - Y E - - - - E V   179
E1   K F S G I L S G S L S Y I F G K - L D E G M S - F S - - - - E A   656
E2   S I S G I F S G T L S W L F L Q - F D G S V P - F T - - - - E L   649

BL   T R - - - - L G Y A E A D P T A D V E G H D A A S K A A I L A S   225
BS   L K E A Q D L G F A E A D P T S D V E G L D A A R K M A I L A R   211
E1   T R L A R E M G Y T E P D P R D D L S G M D V A R K L L I L A R   688
E2   V D Q A W Q Q G L T E P D P R D D L S G K D V S R K L V I L A R   681
```

Fig. 2

BL: *Brevibacterium lactofermentum* BS: *Bacillus subtilis*

E1: *E. coli* HDI E2: *E. coli* HDII

*: amino acid common to three secies

**: amino acid common to four secies

```
        *
BL  I A F H T R V T A D D V Y C E G I S N I S A A D I E - A A Q Q A   256
BS  L - G F S M N V D L E D V K V K G I S Q I T D E D I S F - - - -   238
E1  E T G R E L E L A D I E I E P V L P A E F N A E G D V A A F M A   720
E2  E A G Y N I E P D Q V R V E S L V P A - H C E G G S I D H F F E   712

BL  G H T I - - - - - - - - - - - - K L L A I C E X F T N K E G K S   276
BS  - - - - - - - - - - - - S K R L G Y T M K L I G I A Q R D G S K   258
E1  N L S Q L D D L F A A R V A K A R D E G K V L R Y V G N I D E D   752
E2  N G D E L N E Q M V Q R L E A A R E M G L V L R Y V A R F D A N   744

              *                   * *               *
            * *                 * * *     *         * *
BL  A I S A R V H P T L L P V S H P L A S V N K S F N A I F V E A E   308
BS  I E - V S V Q P T L L P D H H P L S A V H N E F N A V Y V Y G E   289
E1  G V - C R V K I A E V D G N D P L F K V K N G E N A L A F Y S H   783
E2  G K - A R V G Y E A V R E D H P L A S L L P C D N V F A I E S R   775

                        *   * * *       * *           *
                        *   * * *       * *     *     *
BL  A A G R L M F Y - - G N G A G G A P T A S A V L G D V V G A A R   338
BS  A V G E T M F Y - - G P G A G S M P T A T S V V S D L V A V M K   319
E1  Y Y Q P L P L V L R G Y G A G N D V T A A G V F A D L L R T L S   814
E2  W Y R D N P L V I R G P G A G R D V T A G A I Q S D I N R L A Q   806

          * *
BL  N K V H G G R A P G E S T Y A N L P I A D F G E T T T R Y H L D   370
BS  N M R L G V T G N S F V G P Q Y E K N M K S P S D I Y A Q Q F L   351
E1  W K - L G V                                                       817
E2  L - - L                                                           806

BL  M D - V E D R V G V L A E L A S L F S E Q G I S L R T I R Q E E   401
BS  R I H V K D E V G S F S K I T S V F S E R G V S F E K I L Q L P   383

BL  R D D - - - D A R L I V V T H S A L E S D L S R T V E L L K A K   430
BS  I K G H D E L A E I V I V T H H T S E A D F S D I L Q N L N D L   415

BL  P V V K A I N S V I R L E R D                                     445
BS  E V V Q E V K S T Y R V E G N G W S                               433
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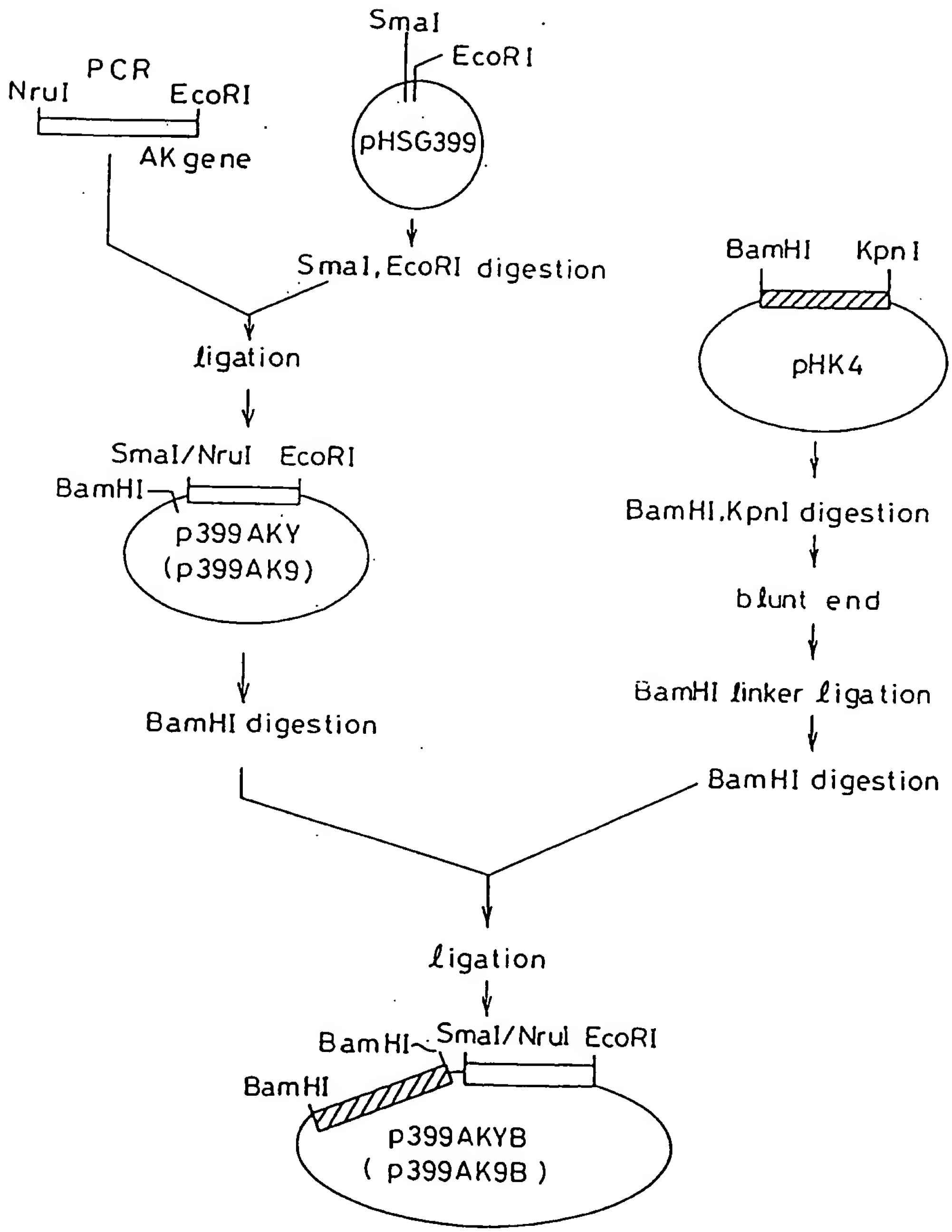
Fig. 3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NruI
EcoRI
AK gene
SmaI
EcoRI
pHSG399
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ligation
SmaI/NruI
EcoRI
BamHI
p399AKY
(p399AK9)
BamHI digestion
BamHI
KpnI
pHK4
BamHI, KpnI digestion
blunt end
BamHI linker ligation
BamHI digestion
ligation
BamHI
SmaI/NruI
EcoRI
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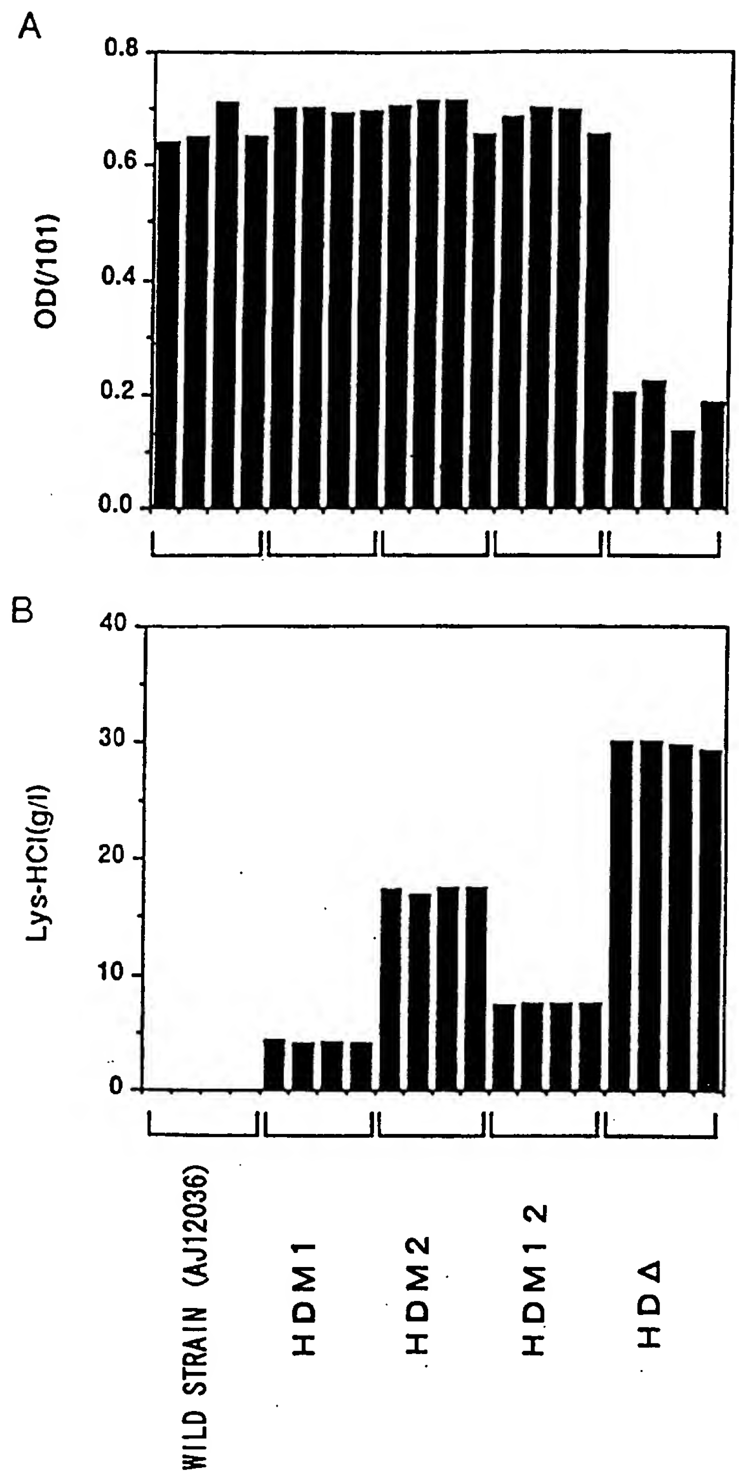
( p399AK9B )

Fig. 4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0.2
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B
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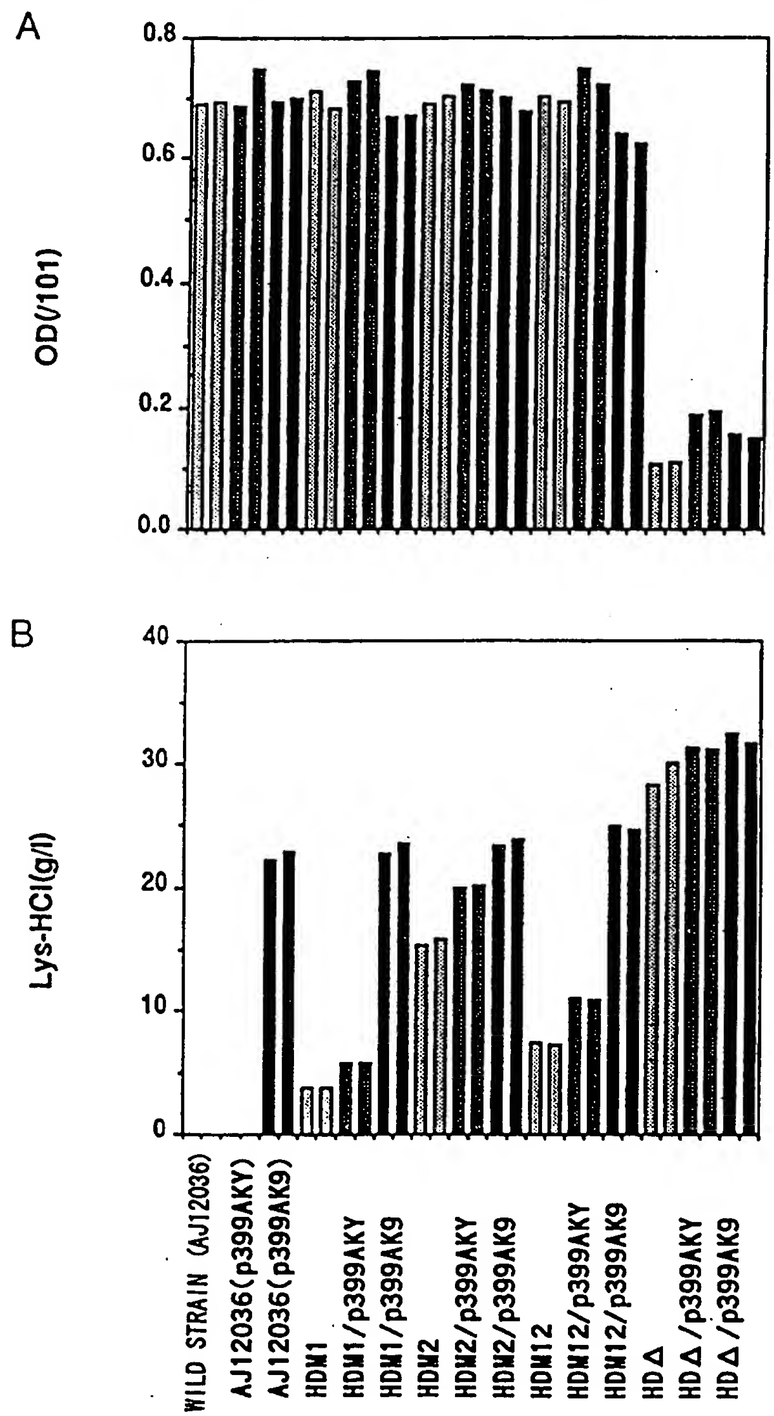
WILD STRAIN (AJ12036)
HDM1
HDM2
HDM12
HDΔ

Fig. 5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10
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WILD STRAIN (AJ12036)
AJ12036(p399AKY)
AJ12036(p399AK9)
HDM1
HDM1/p399AKY
HDM1/p399AK9
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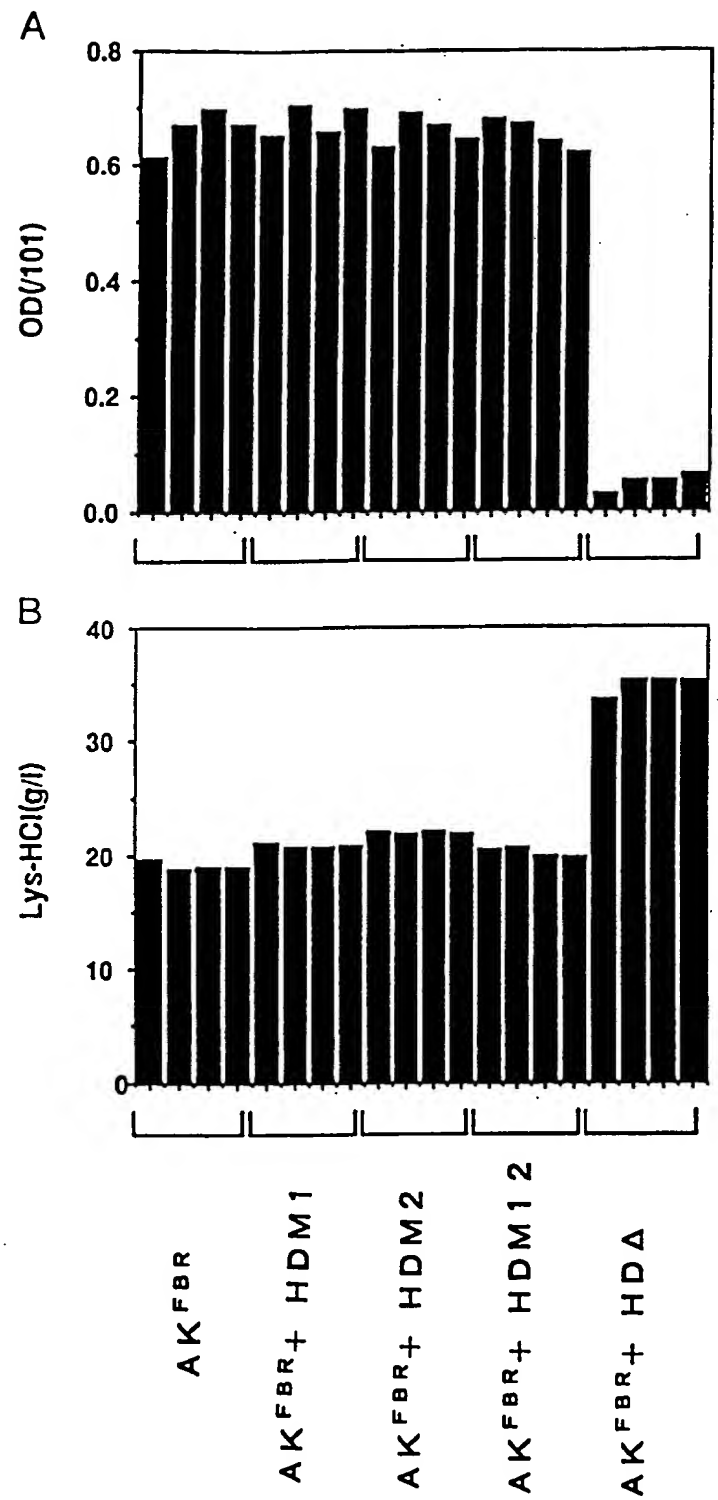
HDΔ/p399AKY
HDΔ/p399AK9

Fig. 6


A
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0.6
0.4
0.2
0.0
OD(/101)
B
40
30
20
10
0
Lys-HCl(g/l)
AK FBR
AK FBR + HDM1
AK FBR + HDM2
AK FBR + HDM12
AK FBR + HDΔ

Fig. 7

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/00268

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. $Cl^6$ C12N15/53, C12P13/08

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Int. $Cl^6$ C12N15/53, C12P13/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS BIOSIS WPI, WPI/L

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y/A | Mol. Microbiol. Vol. 2, No. 1 (1988), Peoples, O. P. et al. "Nucleotide sequence and fine structural analysis of the Corynebacterium glutamicum hom-thr B operon" p. 63-72 | 5, 6, 7, 8/ 1-4, 9-12 |
| Y/A | Mol. Microbiol. Vol. 5, No. 5 (1991), Kalinowski, J. et al. "Genetic biochemical analysis of the aspartokinase from Corynebacterium glutamicum" p. 1197-1204 | 6,7,8/9-12 |
| Y/A | JP, A, 3-219885 (Degussa AG.), September 27, 1991 (27. 09. 91), & EP, A1, 387527 | 6,7,8/12 |
| A | J, Gen. Appl. Microbiol. Vol. 7, No. 3 (1961) Nakayama et al. p. 145-154 | 1 - 12 |
| Y/A | EP, A, 435132 (FORSCHUNGSZENT JUELICH GMBH), July 3, 1991 (03. 07. 91), & DE, A, 3943117 | 6,7,8/9-12 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| April 13, 1995 (13. 04. 95) | May 2, 1995 (02. 05. 95) |
| Name and mailing address of the ISA/ | Authorized officer |
| Japanese Patent Office | |
| Facsimile No. | Telephone No. |

Form PCT/ISA/210 (second sheet) (July 1992)